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TISSUE CULTURE .

TISSUE CULTURE

STUDIES IN EXPERIMENTAL MORPHOLOGY AND
GENERAL PHYSIOLOGY OF TISSUE CELLS IN VITRO

A TEXT-BOOK

BY

ALBERT FISCHER, M. D.

WITH AN INTRODUCTION

BY

ALEXIS CARREL, D.B., B.S., M.D., S.D.

MEMBER OF THE ROCKEFELLER INSTITUTE, NEW YORK

WITH 70 ILLUSTRATIONS AND 1 COLOURED PLATE



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*To the memory of my dear mother and sister
these pages are affectionately dedicated*

INTRODUCTION

The purpose of Doctor *Albert Fischer* in writing this book was chiefly to describe the development of the procedures by which the method of tissue culture is becoming slowly adapted to its main object, the study of the fundamental problems in physiology and pathology. The early technique, which was derived from the beautiful experiments of Harrison and consisted in placing a fragment of fresh tissue in a hanging drop of culture medium, did not allow an accurate analysis of the action of a tissue upon other tissues and upon the humors. The cells were subjected to complex influences, such as those of necrotic cells of their own type, of living and dead cells of other types, and of a medium which deteriorated spontaneously in a short time. It was as impossible to study cell physiology with a fragment of tissue of complex composition as to investigate the properties of bacteria living in a medium contaminated by other bacteria. The earlier techniques led to many errors, and had to be profoundly modified before the method of tissue culture could become an instrument of physiological investigation.

The advances made during the last few years, which Doctor *Fischer* has so well summarized, have been rendered possible primarily by the isolation of pure strains of cells and by the development of procedures for measuring the rate of growth and for studying the functional activity of the tissues. The use of pure strains of cells instead of fragments of fresh tissues has been of fundamental importance, and Doctor *Fischer* has contributed to this progress by obtaining the first permanent strain of epithelial cells. Although the technical improvements realized recently have been considerable, the method is still in its infancy. There is need of many experimenters in this field. The book by Doctor *Fischer* will be of particular value to those who desire to use the modern techniques in tissue culture, and will contribute markedly to the progression of an important method of investigation.

New York, February 6, 1925.

Alexis Carrel.

AUTHOR'S PREFACE.

This work was carried out in the Department for Experimental Surgery of The Rockefeller Institute in New York 1920—22, and continued in The Institute for General Pathology of the University of Copenhagen.

I wish to acknowledge my gratitude to the director of the laboratories of The Rockefeller Institute, Dr. Simon Flexner, for his aid and hospitality during my stay at The Institute, and I wish to express my great indebtedness and high esteem to the Chief of The Department for Experimental Surgery of The Rockefeller Institute, Dr. Alexis Carrel, whose incentive, broad scientific views I shall always admire.

Likewise I wish to express my great appreciation to the Director of the Institute for General Pathology of the University of Copenhagen, Prof. Dr. Oluf Thomsen for his kindness and assistance, which made it possible for me to continue the work under such good conditions at a time when, for scientific research in general, these were not of the most favourable.

Copenhagen, Marts 1925.

A. F.

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I.

INTRODUCTORY REMARKS.

It has been intended by this monograph to build up a fairly complete history and criticism of the work which has been done on tissue cultures, and in consideration of the difficulties in writing a book like this on a field of investigation which is progressing so intensively, all possible reservation has to be taken. In the introduction will be given a short historical review — and the history of the special themes will be dealt with in the various chapters. The contributions and experiences made by myself are not kept separately, but are added to the chapters to which they belong in order not to break up the continuity of the book.

The history of the tissue cultures is strictly speaking not more than 15 years old — and to-day there seems to be only a relatively limited understanding of the importance and significance of the tissue cultivation as a method for making elementary and fundamental studies of the physiology and pathology of higher organisms.

The one-celled organism has been studied and submitted to a thorough investigation in the belief, that a simple organism is easier to understand, but things seem to prove that the one-celled organism is not so simple. In the highly differentiated organism the circumstances are entirely different and probably simpler because we perceive some kinds of elements (cellgroups or organs) and to a certain extent understand their work. Nevertheless the physiological and pathological processes are very profound and complicated matters owing to the many unknown fac-

factors which are encountered. Physico-chemical processes are continuously going on in the body-juices as a result of the various tissues. In the advanced technique of cultivating animal tissue in vitro, a first step is made to submit the differentiated organism to a causal analytic investigation. By introducing the histological physiology into experimental medicine and biology we have a new era. The building-stones of the organism can now be investigated under such experimental conditions, that many of the complicated factors, which exist in the organism, can be eliminated or reduced in the culture.

Among the numerous problems to be investigated by this line of research, it seems to us very important to know a little more than we do already of the constitutional laws determining the regulation of growth of the various tissues and organs.

II. HISTORICAL REVIEW.

Normal physiology has nearly always dealt with the investigation of the functions of organ-systems, single organs or certain important cell groups in the body without going into the study of the physiology of the tissues in general. We must give the pathologists the credit for the initial studies on the various tissues. Up to the present more contributions to the normal physiology of tissues in general have been accomplished by the pathologists. When *Virchow* created the cellular pathology, new points of view were opened up and new fields for investigations were fertilized.

The methods of explantation applied in the studies of tissues were very extensively employed in the new cellular pathology and many exceedingly important problems were solved. The biology, or general physiology, included mostly the study of the lower organisms, protozoa, etc., but normal physiology made use of the methods of explantation too, — mostly however of organ-systems, as for instance the study of the action of the isolated heart, kidney, intestines, and so on. But, as already mentioned, the morphological experiments on explanted tissues were actually inaugurated by the pathologists.

The subjects for investigation by the methods of explantation were, to begin with, usually blood cells, egg cells, spermatozoa, ciliated epithelial cells, cells which were easy to isolate and observe *in vitro*. Later on transparent organs such as the cornea, thin membranes and similar structures were studied *in vitro*. The experiments of *Cohnheim*

on excised cornea will be remembered, by which method he endeavoured to solve the most fundamental problems of inflammation. Most of these experiments were not actual explantations, often the cells, which were to undergo an examination were in some way connected with the body; for instance the many experiments of inflammation on the mesenterium, *membrana natatoria* of frogs, were mostly done in connection with the animal. In other cases the animal was generally used as an incubator for the cells, as, e. g., in the experiments on chemotaxis, when for instance, white blood corpuscles were tempted to wander into capillary tubes, placed in the abdominal cavity and containing attractive substances for the cells. In studying the rôle played by the connective tissue cells under conditions of inflammation, Maximoff placed small ground glass plates in the peritoneal cavity and afterwards examined the invaded inflammatory tissue.

It has been known that dead material lying in the organism, as for instance masses of fibrin in the form of thrombi in the blood vessels, can be organized and progressively brought to disappear. What is going on is namely that leucocytes and connective tissue cells migrate into the fibrin and live there until the fibrin is liquified and resolved. In other words, we here actually have a tissue culture *in vivo*. Experiments resembling this process have been carried out by Leo Loeb²⁷² in 1897. He observed the cell-wandering in blood coagulum and agar *in vivo* and claimed also to have done it *in vitro*, but the description of the technique has not been published.

Only when Harrison²⁴⁶ in 1907 succeeded in studying the regeneration of nerves from frog embryos in lymph outside the organism was this field of investigation opened. Harrison's method of investigation was a logical outcome of the embryological method of Roux, Driesch and Born, in which they tested the isolated embryonic parts in regard to their function in the formation of organisms.

Carrel foresaw in this method a very important way to make a fundamental study of tissue cells under various experimental conditions and together with Burrows he extended the use of the method to the cultivation of other kinds of tissues. By an ingenious method of organizing the experiments a marvellous technique was developed for tissue cultivation in vitro. Carrel and Burrows⁷¹⁾ very soon succeeded in cultivating all kinds of embryonic tissue, tissue from different adult organisms and cells from neoplasms. The results of these experiments were looked upon by the scientific world with great expectations. The experiments were repeated by the majority of biologists, morphologists and pathologists, without, however, much success and with rather disappointing results.

Consequently many investigators became very sceptic and pessimistic in regard to the employment of the method. The progress in this line of work by the pioneers was so great that it was hard for other investigators to keep pace with it and they became sceptics. A large quantity of literature was published on the subject, and from France and Germany especially, a decided opposition was met with. This part of the history is described very dramatically in Albert Oppel's¹³²⁾ book on explantation, to which can be referred. The objections generally urged were, that the apparent growth observed in the tissue cultures was not actual growth, but a combination of active cell migration and a passive outfloating of cells caused by currents from the explanted tissue to the medium.

Some investigators went so far as to regard the growth as a phenomenon of necrobiosis. (Jolly's^{276), 277}). The reason why so much opposition was encountered was primarily that the organization of the culture work made by others than the pioneers was very poor and that from few unsuccessful experiments unreliable conclusions were usually drawn. It was necessary accurately to follow up the technique if good results were to be expected and this particular work requires much care and patience if success is to be expected.

It is remarkable how completely the technique was worked out from the very beginning. This can be seen by all the experiments which have later been attempted, for instance, to endeavour to modify the culture medium, to obtain another supporting apparatus for the tissue cells to grow on. All that has been tested as a substitute for one or another of the constituents of the plasma-tissue juice medium, has been so far without success. It seems that the only satisfactory method was accomplished from the very outset.

In practically all branches of experimental medicine and biology the method of cultivating tissue has been applied already. As a morphological and physiological method, the tissue cultivation method of explantation was introduced when R. G. Harrison ²⁴⁹⁾ solved the fundamental question of nerve regeneration as an active cell movement and growth of the axis cylinders by studying the phenomenon in vitro. After these successful experiments several morphologists took up the method for the study of other problems. Studies of cell divisions, cell degenerations, were undertaken. The histogeny of the various cell products, as for example connective tissue fibrils, was studied. This can be referred to in the works of Lewis and Lewis, Maximoff, L. Loeb, Baitzell, Lambert and Hanes, Uhlenhuth, Oppel, Champy and several others.

It seems to be the morphology which has interested most investigators: probably because of the less difficult technique for making morphological studies. An explanted fragment of tissue of any kind is a rather easy matter to keep living in vitro for a short period from a few hours to several days and one can still observe an outgrowth or migration of cells, besides some active cell divisions. For those experiments of short duration, the composition of the culture medium does not play any important rôle. As Lewis and Lewis ^{327), 349), 350)} have shown, an outgrowth of cells can be observed in a simple fluid con-

taining only inorganic salts (Ringer's solution or seawater) and the surface of the cover slip can be used as the only supporting apparatus for the wandering of the cells. This method cannot actually be called tissue cultivation. It is survival only of fragments of tissue in vitro, a survival in a protective solution which is not a growth-promoting, nutrient solution. The tissue cells live and multiply at the expense of the nourishing and growth-promoting substances contained in the explanted piece of tissue.

It is perfectly true, as Burrows has stated, that fresh embryonic tissue grows not because of the medium, but in spite of the medium. The disintegration of some cells liberates growth-promoting substances for other cells, naturally within certain limits. The only true cultivation of tissue is the method which deals with permanent strains of various tissues. Then we can speak of an actual growth in vitro, the advantages of this method being namely, that the conditions of the experiments are much simpler and more constant. The permanent strain is pure and under uniform conditions all the time and not influenced by the many factors of the organism which are still there immediately after explantation. To learn anything about the influences of the various components of the culture medium, or whenever certain problems have to be investigated, it is necessary to carry on the strains over a certain protracted period of time to be sure that the explanted tissue has been adapted to the new experimental environment. The above-mentioned experiments of Lewis and Lewis,³²⁷ for instance, demonstrate the fact that fresh explanted tissue is able to live in simple salt solutions and grow for a very short time at the expense of the substances in the tissue itself, but when it is attempted to transfer the tissue from one salt solution to another it usually results in the death of the tissue. Consequently it is rather confusing when investigators, who have not worked

much in this line before, state that, for example, tissue can be cultivated in so simple a medium as "sea-water".

It is important that some distinction be made between the two methods of investigation, the one which deals with experiments on surviving tissue in protective solutions and the other one, the *in vitro* cultivation of tissue in culture media which actually promote the growth.

This fact seemed to be very little understood by several investigators; only very few people work with tissue cultivation in a confident manner, whatever may be the cause: lack of technical difficulties or understanding.

In studying physiological and pathological problems it is therefore of great importance to carry on the experiments over a long period of time and to use permanent strains of tissues which have acquired a certain grade of stability for the *in vitro* life. It was taken for granted at the early period, when the technique for tissue cultivation came up, that the blood plasma contained all the substances for the nourishment of the tissue in the culture. Of course there are some; but experiments have recently shown that the plasma or serum has a more inhibitory than a growth promoting influence (Carrel and Ebeling) ⁽¹⁰¹⁾ and this fact could only be abstracted from a method which has the purpose of following the rate of growth over a long period of time. And the same method resulted also in finding those substances which are absolutely required to make the tissue live indefinitely. It was early found by Carrel ^{(48) (58)} that the juice from young embryos contain all the necessary substances for the indefinite life and that tissue juice and blood plasma from adult and old individuals do not contain these substances in any amount sufficient for the indefinite life. In this connection it may be mentioned that the tissue cells from young and old animals can be cultivated indefinitely, i. e., that it seems as if the factors of senescence are not to be found in

the cells themselves, but are far more profound phenomena correlated to the entire function of all the cells in the organism and their "milieu interieure" (Carrel) ⁷³.

The evident significance of this method was foreseen very early by Carrel and a technique was developed by which the rate of growth of the tissue in vitro could be measured. Carrel and Ebeling ¹⁶³⁾ worked out the quantitative study of the growth of fibroblasts in vitro. In regard to the imperfectness of the method and respecting the more or less complicated phenomena dealt with, by measuring the surface area of growth, the accuracy of the method was found to be sufficient to discover new facts and the first important step was made for studying physiological phenomena quantitatively.

In order to develop a fairly safe technique one has to have a more than ordinary interest for the problems and a good deal of patience. The technique is not so difficult; almost any person can be trained to do it. It is shown by Ebeling ¹⁶³⁾ that if a culture is divided into two equal parts and cultivated separately under uniform conditions, the rate of growth does not vary more than 8-10 per cent, which must be said to be very accurate in regard to the imperfectness and the complexity of the method.

By employing the quantitative method very interesting and important discoveries have already been made and by this method unlimited problems of importance can now be attacked. For example, the problems of senescence have been investigated by Carrel; later Carrel and Ebeling ^{92, 94, 102)} have shown that in plasma an inhibitory factor for the growth of connective tissue is contained, and that cells grown in plasma of younger animals grow much more abundantly than in that of older animals. The effect of age is marked, the rate of growth of fibroblasts in chicken plasma decreasing 50 per cent with the first three years of life and 30 per cent more in the next six years of the life of the fowls furnishing the plasma. And they

have called attention to the fact that something similar must apply in man since the rate of cicatrization of human wounds, measured accurately, is found to vary inversely with the age of the patient by applying the du Noüy's ¹²⁰⁾ formular for cicatrization of wounds to the rate of growth of the fibroblasts.

The changes in rate of growth seem to be one of the most important responses of the various tissues in culture when the conditions are somewhat changed, especially when the changes are of a chemical or biochemical nature. Changes in the composition of the culture medium, adding bacterial proteins, or treating the various compounds by heat or chemicals, and so on, result apparently only in changes in the rate of growth and apparently not in changes in morphology, at least not to a noticeable extent ^{190), 198)}. Therefore the determination of rate of growth becomes highly important. It seems that when changes in the culture medium are of a more mechanical nature morphological changes in the cells occur, L. Loeb, ^{373), 378)} Uhlenbuth ⁴⁹⁸⁾.

It has already been observed by Cohnheim that the leucocytes when they appear in the cornea under conditions of inflammation become spindle-shaped and fusiform as fibroblasts. Later Leo Loeb ³⁷⁴⁾ observed in epithelial cells similar changes when, under experimental conditions, he examined the migration of these cells in blood coagulum or agar. Recently E. Uhlenbuth ⁴⁹⁹⁾ studied the morphological changes in epithelial cells from frogs when cultivated in culture media of varying consistency. The changes in shape of the cells show that the mechanical factor plays an important rôle here. The leucocytes in the inflamed cornea become elongated on account of the mechanical structure of the cornea; the same thing happens to epithelial cells in the dense fibrin meshwork in the clot. No doubt the outer appearance of the tissue cells depends mainly on the architecture of the sup-

porting material, and it is important to notice how determining the stroma is for the morphology of cells. It is absolutely essential for the life of tissue cells to have a solid, or in some way solid support for their outgrowth.

This phenomenon of the necessity for the tissue cells to have a support or a frame work for retaining the life has been discussed by Harrison ²⁵¹. The support may be the fibrin in clotted plasma, the surface of the cover glass, the surface of the fluid film, the framework of spider-webs, silk gauze or cotton threads or the like. In cultures in which the tissue floats free in a fluid medium no outgrowth occurs and the cells succumb. The cells, cultivated in a fluid with the cover glass as the only support tend to be much more flattened because there are only 2 dimensions to the support: when cultivated in the fibrin clot the cells are fusiform and more tubular because of the three-dimension support.

Lee Loeb called this dependency of the cells to the solid phase, stereotropism. If cells are permitted to live in a fluid medium they will succumb very rapidly. It is the solid supporting apparatus which determines the form of the tissue cells, and without support the cells contract themselves, become round and seem to be unable to assimilate and multiply. This phenomenon that the cells become round as soon as the culture medium liquefies or when the supporting apparatus is removed has probably been observed by several investigators, but was first described by Uhlenhuth ⁴⁹⁸, later by Rous ⁴⁵⁷ and then by myself ¹⁹⁸⁵. It is probably a phenomenon which can be explained by surface tension, as Bütschli ⁴² and Quincke ⁴⁴⁷ have explained similar phenomena.

Under the conditions of experiments generally used, we do not actually find morphological changes, transformation or differentiations in the cells cultivated in vitro. The chemical and mechanical environments are usually the same all the time in the cultures. The age phenomena of the

cells are suppressed owing to the fact, that the culture medium always contains substances from an organism in early embryonic stage. Age and differentiation are probably related phenomena, very complex, due to co-operation between all the organ systems. Much discussion has appeared as to whether cells from the various tissues dedifferentiate when cultivated in vitro to primitive embryonic, indifferent cells, as stated, for instance, by Champy¹²³ and later by Uhlenhuth¹²⁹. Oppel¹³² mentions in his book that tissue cultivation is a disappointment owing to the fact, that the outgrowing cells are indifferent and cannot be qualified. All investigators agreed with him at that time. It is, however, not true that tissue cells dedifferentiate in vitro; I have demonstrated that epithelial cells, when they are obtained pure, free from other cells, grow in vitro indefinitely, in a manner characteristic for epithelium, and no transformation or dedifferentiation takes place. In these experiments it is important to get from the very beginning the pure tissue for cultivation, equally as important as it is when cultivating bacteria. In obtaining pure cultures of the various tissues for cultivation, unfortunately the method used in bacteriology can not very well be applied here. The safest and best method is to start out with tissues which are pure when taken out of the body; or take advantage of the fact that certain tissues grow better in vitro than certain others: this physiological elective method can with success be employed in getting epithelium from the iris attached to the lens or as W. H. Lewis* has indicated to cultivate pieces of amnion which consist only of smooth muscle and epithelium. Neither the lens nor the smooth muscle grow for any length of time and progressively the epithelium is obtained pure. But the very best and most reliable method is to get the tissue pure from the very beginning from certain loci electi. Cartilage has been obtained pure in a like manner.

*) personal communication.

It was declared in the early period of tissue-cultivation, that all cells cultivated in vitro, dedifferentiated to a primitive indifferent cell type; it can therefore be stated as a very important and fundamental fact that ectoderm and mesenchyme do not change their character. This seems to indicate that probably the physiological character of the cells also should remain constant. This fact, certainly, gives us an opportunity to investigate the physiological characteristics of the various tissue cells outside the body and perhaps elucidate the more or less antagonistic cytological interactions between the various types of tissue cells in the organism.

The problems to be investigated by the tissue-cultivation method are unlimited and, as mentioned before, if used in a proper critical manner, it is the most rational method for the study of physiology and pathology. During the short time that this method has been propagated into the many different lines of biology, many more or less hazardous conclusions have been brought out, mostly built on very imperfect technique.

The histogeny of the various intercellular substances, such as the different fibrillae of the connective tissue, the substances which link the cells together in cartilage and bones, etc., will find a good method for investigation in the tissue cultivation. The old question of the origin of the connective tissue fibrils was taken up by Baitsell^{7, 8, 9} and later by M. Lewis³³⁹ by the tissue-culture method. Baitsell represents the idea that the fibrils have their origin in the plasma and can be reproduced without the presence of living cells, but by mere mechanical factors; he explains it as a transformation of the amieronic fibrin particles in the plasma and probably under the influence of the different enzymes in the blood. It is known from the chemistry of colloids that different mechanical factors have a denaturing effect on the colloidal solutions and without doubt the same phenomenon is dealt with here. On the contrary M. Lewis

observed in a fibrin-free medium that the mesenchyme produces fine contractile fibrils after twenty-four hours of cultivation and by staining for mitochondria these could be found present in the fine fibrils.

Concerning the cartilage ¹⁹¹, there seem to be interesting processes to study in regard to the intercellular substance, which seems to disappear when the cartilage is cultivated embedded in the plasma clot and the cells fall apart and resemble a culture of yeast, but cultivated on the free surface of the plasma clot, the cells spread out, grow as do epithelial cells, adhering in solid membranes. It can now be considered as a fact that cartilage, as well as epithelium can be brought to live outside the organism for any length of time.

Another question which has caused an endless and life-long discussion viz: whether the invading cells under conditions of inflammation are derived from Marchand's leucocytoïd cells or from Maximoff's polyblasts, seems in the culture work to stand an opportunity of being solved. v. Recklinghausen showed that the pus cells and white blood cells were the same thing and Cohnheim observed that the white cells wandered out from the vessels under conditions of inflammation. His ingenious experiments on the excised cornea will be remembered.

It is well known what an incessant and life-long struggle P. Grawitz ²³⁰ had because of his statement that pus cells are derived from the fixed tissue cells when their respective supporting substance is destroyed. Doubtless his statements are not absurd. Leo Loeb's ³⁷³ experiments on the regeneration of epithelium show that the infiltration could be explained that way. In recent investigations on growing cartilage in vitro ¹⁹⁴, we have an example which shows how the intercellular substance can be brought to disappear under certain conditions. The changes of any tissue cell into the spherical shape can be seen any day in the tissue cultures. The studies of the origin of the diffe-

rent hematogenic elements, will probably also some day be cleared up by the method of explanation.

Another big unknown field in the physiology and pathology of tissues, which doubtless can be accomplished through the cultivation method, is the study of the intimate structure of the cell under various conditions, the study of the degenerative processes as well as the generative processes in the cells. The biochemical investigation of the conditions under which mitochondria, fat, glycogen and pigment are produced and the conditions under which the various degenerative processes (amyloid for instance) take place can be studied in the tissue cultures.

Within the pathology, the work on immunity will be exceedingly important from the viewpoint of tissue cultivation. To observe, for instance, whether there are certain tissues only which contribute to the formation of antibodies, or whether there are certain tissues which possess a natural immunity to certain virus; not to speak of the enormously interesting field of the cytotoxins. It is difficult to understand why not more investigators have taken up the study of immunity in tissue cultures. Carrel and Ingebrigtsen⁹⁰⁾ and later Fischer¹⁹³⁾ 196) are the only ones who have studied the production of antibodies in tissues cultivated in vitro. Burrows used the growth of tissues in vitro as an indicator for the toxin and antitoxin combination. This has actually nothing to do with the production of antibodies in vitro. A. Fischer¹⁹³⁾ 196) has shown how it is possible to use the tissue cultures to study the action of antigen and, at the same time, in its rate of growth obtain a measure for the amount of antibodies produced during a period of immunization. By this method it will be possible to see if all kinds of cells respond in the same way, as did, for instance, the ten-year old strain of fibroblasts. Through this immunological method it will probably be possible to study the interaction between the various differentiated tissue cells. In other words, to investigate the

conditions, chemical or physical, which result in a progressive disappearance of the epithelium in cultures, when fibroblasts are present. Is the cause to be found in the cells themselves, in a certain difference in the physiological character of the two cell types, or in the condition of the culture-media?

One would imagine that experimental radiology would have an exceedingly good method in tissue cultivation for studying the ray energy on the various isolated tissue cells. In short, there can scarcely be found any problem in experimental medicine that could not take advantage of the tissue cultivation method, especially now when it has been shown that also other tissues can be grown pure in vitro, and, what is more important, that a dedifferentiation of these tissues does not occur under the experimental conditions; but that they remain in the same state of development as they were in the animal from which the cells and the tissue juice necessary for the growth are taken.

When the technique for cultivating tissue cells was developed, several investigators expected much of the method to study the character of the malignant cells.

The question was therefore quickly taken up by most investigators, but the results were rather disappointing. Already as early as in 1910, shortly after the technique has been developed, Carrel and Burrows,^{76, 77, 78,} later Lambert and Hanes^{505, 507,} worked intensely on the cultivation of malignant tumours in vitro. — The technical difficulties for keeping the tumour cells growing over long periods of time were rather great. It was stated by Carrel and Burrows, that no actual characteristics were found, that were not also found of some normal tissue cells. Not until within the last few years these questions have again been taken up by several investigators.

The realization of many of the said problems will mainly depend upon an improvement in the technique, an understanding of the value of this line of investigation and the

knowledge of the limitations of the method among the investigators. It must be repeated that to get a thorough understanding of the physiology and pathology of tissues, it is important not only to keep the cells surviving in vitro, but is more important to deal with permanent strains of the various tissue cells in order to operate with more clearly cut experiments. First then we may expect that this method will bring clearness to the many mysterious problems, hitherto unassailable because of the complexity of the organism as a whole.

III. CULTURE MEDIA.

The medium necessary for the cultivation of tissue cells outside the body can be separated into two main components, the supporting apparatus or framework and the growth-promoting substances.

It has already been found by several investigators that fragments of tissue transferred to a liquid medium die very soon. It is therefore of vital importance that the cells find some kind of solids to migrate on, if not, the cells become spherical and succumb progressively. Leo Loeb³⁷⁴ has the credit for being the first to study the influence of the solid phase for the wandering of the tissue cells, and at the same time, indicated the mechanism of the cell migration in regard to the solids. Later Harrison²⁵¹ showed the necessity of the framework in the culture medium for the growth of nerve cells. E. Uhlenhuth⁴⁹⁸, P. Rous⁴⁵⁷ and myself⁴⁹⁸ observed the changes of the different cell types into spherical forms, when the coagulated culture medium became liquid. It is consequently necessary to have some kind of support for the cells in the cultures. When Harrison²⁵¹ studied the nerve regeneration *in vitro*, he only found growth when using clotted frog lymph; if it remained liquid no growth took place. When Burrows modified the technique by using blood plasma instead of Harrison's lymph, the most ideal culture-medium for tissue cells was found. It is ideal because it is the most natural condition for the cells and because it allows a

thorough microscopical examination on account of its perfect transparency.

Frequently it has been endeavoured to modify the culture medium to make it simpler, i. e., to get a supporting material of a more indifferent character and of better known composition than the fibrin meshwork. Agar, gelatin, glass wool, silk gauze, spider webs, cotton threads, etc., have been tried, and all can be used as a framework for the outgrowing cells, but none of them is able to give support for as uniform an outgrowth as does the fibrin. When it so often is referred to that Lewis and Lewis use so simple a culture medium as Ringer's solution or even seawater, it is somewhat confusing. The outgrowth finds its support on the coverslip itself and the tissue grows for a short time at the expense of the growth-promoting substances in the tissue fragment itself. These experiments are often referred to, to show how simple it is to cultivate tissue. For the inexperienced it may therefore be understood as though the cells can be cultivated in a liquid and without any nourishing substances. The use of the surface of the coverslip as a supporting apparatus is very good so far, but it is not a safe method to get outgrowth for any length of time. To get a growth on the cover slip or any other two-dimensioned support, it depends very largely on the contact of the tissue with the support: mostly those cells which are in direct contact with the glass will migrate. It is different when the tissue is imbedded in the plasma clot and perfectly surrounded by a fine meshwork: then there will always be a sufficient and good contact between the cells and the supporting apparatus. The fibrin clot can be considered as a fine sponge of fibrin threads holding the liquid in its meshwork. In between these two phases of liquid and solid, the tissue cells migrate.

This fibrin system seems to be unsurpassed as a culture medium for the cells. The fibrin-threads do not show up in the microscope and it is therefore an excellent transpa-

rent gel of such a fibrillary construction that will be difficult to imitate in any other way.

It has often been essayed to use either gelatin or agar in tissue cultures and it has been frequently stated in the literature that the tissue cells will grow in agar. Leo Loeb³⁷² claimed that he observed, for instance, an ingrowth of different tissue cells in agar *in vivo* and *in vitro*. The Lewis's, Ingebrigtsen²⁶⁹ and others observed the same thing.

In experiments made by myself* in trying to cultivate fibroblasts *in vitro* in agar and gelatin, I found that the tissue cells are unable to grow in agar or in agar mixed with embryonic tissue juices or serum as Ingebrigtsen²⁶⁹ observed it. The agar material is of quite another structure than the fibrin coagulum. Agar is a dense, impenetrable mass, not of the same spongy nature as the fibrin clot, but of agar hydrate nature: the water is much more closely bound and can not, for instance, be squeezed out as in the case of the fibrin coagulum. I have often seen the growth of fibroblasts in agar and different mixtures of agar and other substances, but by careful observation I always found the growth as a surface growth in between the solid agar surface and the liquid, whatever it may be, serum or tissue juice: or I could observe a growth apparently in the agar, but it was found to occur only when the agar mass was broken into pieces and the cells were then found to be located in between the solid agar and the fluid serum. In Ingebrigtsen's²⁶⁹ experiments agar was mixed with serum at a temperature of 50°C. for instance, and at that temperature the fluid agar does not form a suitable mixture with a liquid, the agar will then consist more or less of lumps of agar and fluid apparently mixed. This experiment and interpretation of this artificial growth of tissue cells in agar suggested, that if we could make an artificial

*) not published.

mixture of gelatine and agar, i. e., agar which, for instance, contained gelatin evenly and mechanically distributed, then probably the tissue cells could be successfully cultivated. The idea was, that at the temperature of the incubator, 39°C, the gelatin would become fluid in a meshwork of agar and thus allow the cells to grow in between the liquid gelatin and solid agar. These experiments were, however, not at all successful.

From these experiments it would be concluded that it is not true that growth of tissue cells can take place in agar as such, only on the free surface under cover of a liquid film. The explanation, therefore, why several investigators such as Leo Loeb, Lewis and Ingebrigtsen have seen growth in agar is simply, that they have taken the surface growth for an infiltration growth in the agar itself. Leo Loeb describes the penetration of epithelial cells, for instance, in the agar; the agar has probably, in the *in vivo* experiments, got some more or less minute cracks into which the lymph has been soaked and therefore created a medium for the ingrowth of the cells. I then tried to crush the clotted agar, and by adding a liquid such as embryonic tissue juice or serum to the fine agar particles, a growth could be obtained. This method did not seem to have any advantages because of the much slighter transparency of the medium for microscopical purposes and the uneven growth.

It was found in my experiments that the quantity of liquid added to the agar particles was most determining for the outgrowth of cells. If there was an excess of liquid no growth took place, but if there was added just enough to cause the liquid to stick by capillary suction to the surface of the particles, an outgrowth of cells occurred. The same phenomenon was later observed by using other solids as a supporting apparatus (cotton, glass-wool). There seems to be an important connection with the surface forces and the migration of cells which also has been observed by other investi-

gators. The surface of a liquid is sufficient support for the migration of cells. This phenomenon can be seen very often while working with tissue cultures. It can be seen how tissue cells are able to grow on the free surface of the liquid.

The growth of tissue cells is closely connected with surfaces, and the fibrin clot can therefore be considered as an enormous surface of the fibrin covered with a thin film of liquid by means of capillary suction.

In some other experiments I tried elderberry pith as a support for the growth of fibroblasts. Thin discs of the pith were brought to float in the liquid embryonic tissue juice and the tissue fragment was placed on this moist surface. Growth took place on this surface quite extensively and the disc was removed from the tissue juice after 48 hours in the incubator, washed in Ringer's solution and made to float again on fresh juice. In this way the tissue could be kept alive for a long period merely by changing the liquid medium. In the meantime the pith did not prove to be very good for microscopic examinations; it had to be sectioned and stained if it was to be subjected to a thorough microscopic examination. The utility of this method would, perhaps, be found when it is necessary to grow tissue on the same supporting apparatus for a long period to study a possible changing organization during a constant renewal of the nourishing and growth-promoting substances.

Other materials have been tried as frame work for the tissue growth. Harrison²⁵¹ used spider-web; Carrel used silk gauze and hair and I have used ordinary adsorbent cotton. All these substances can be successfully used as a framework but the outgrowth always depends on the primary contact between the tissue fragment and the material. For a quantitative study of the rate of growth these substances cannot very well be used because of the many incidental errors.

To get a good growth on these substances, it is important to take two things into consideration, namely the contact between the tissue and its support and the amount of fluid; just enough fluid that can be held in capillary suction of the supporting material, gives the best results. It looks, as if the cells are forced to migrate when they are under influence of capillary or surface activity.

The significance of finding other substances which can be substituted for the fibrin in the culture medium is evident. Under conditions of experiment it is often necessary to treat the culture medium in ways which would affect the coagulation of the plasma. Some methods of treatment result in the coagulation not taking place, and consequently no outgrowth occurs. Therefore, we are at present in that respect limited in our study of the growth phenomena to such conditions only which do not interfere with the coagulating process. A typical example can be brought to notice here. It is impossible to study the rate of growth in culture media with a hydrogen ion concentration over Ph. 5 and below Ph. 8.5¹⁹⁰. Only within the limit of Ph. 5—8.5 a fairly good coagulation takes place. Therefore it would be important to find another supporting substance for the growth of tissue cells when such problems are to be investigated.

In making several attempts, and especially by using cotton threads as supporting material¹⁹⁸, very interesting observations were made. There is no difficulty in getting a good outgrowth of cells by using a little piece of absorbent cotton and a drop of embryonic tissue juice. The cells grow out on the cotton threads and often the growth may be so extensive that the open meshes in between the threads are filled up with cells, and by careful examination it is found that the cells grow on the liquid film which by surface tension is held in the meshes. (Fig 1. The experiments mentioned here, suggested that if the cotton threads

could be made so short that they only would contain one or a few cells, these cultures could be used for obtaining pure cultures from one single cell. In other words, the growth on fine cut cotton threads made it possible to aspirate the whole liquid culture and distribute the small cotton pieces

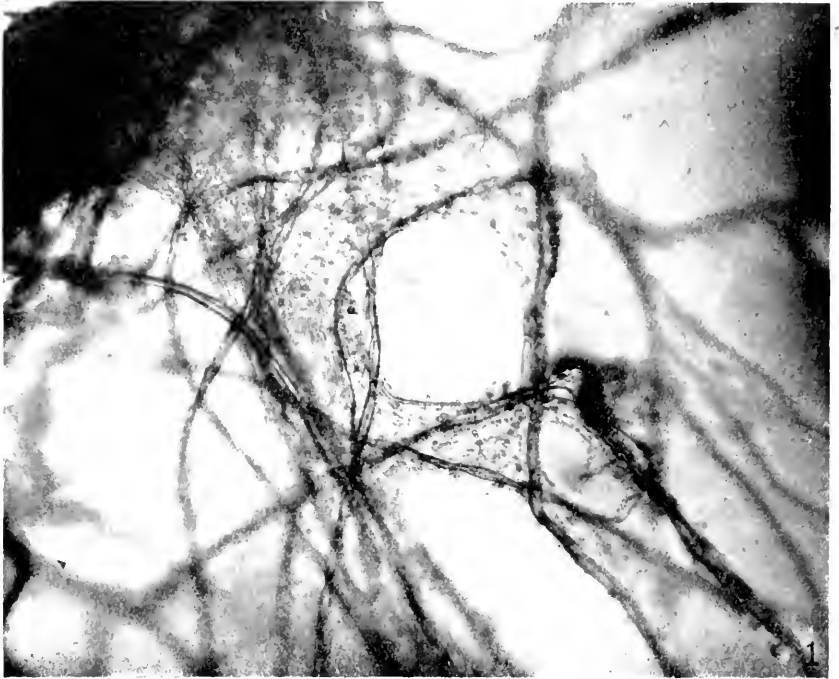


Fig. 1.

Fibroblasts growing in a meshwork of cotton threads in a drop of embryonic tissue juice. From Journ. of Exp. Med. 1923, XXXVIII. 667.

including the growing cells, in a fixed medium, thereby obtaining colonies of tissue cells as is done with bacteria. I succeeded very well in getting isolated cells in that way, but an outgrowth of new cells from a single individual was never observed. This phenomenon will be discussed in

another chapter. It may be mentioned here, however, in connection with the discussion of the relation of solid framework to the cell growth and the cell shape, that as soon as the cotton threads are aspirated, the remaining cells in part of the drop of tissue juice on the coverslip immediately become spherical. The remaining cells contracted and agglutinated to an aggregation of round cells and looked like leucocytes. Also, the cells aspirated in the pipette and distributed in the plasma which later coagulated were all spherical, but nevertheless alive, with a lively amoeboid movement and they appeared later in the clot as typically spindle-shaped fibroblasts.

THE GROWTH PROMOTING SUBSTANCES.

It has been known for a long time (Carrel, 1913⁵⁸) that the culture medium consisting of coagulated plasma only, was not sufficient to keep the tissue cells alive in vitro during long periods and just recently Carrel and Ebeling (1921^{92, 93}) have shown that even the homologous serum has an inhibiting influence on the rate of growth of fibroblasts. In other words, the indefinite multiplication of fibroblasts is due neither to the fibrin nor the serum, but to substances contained in the embryonic tissue juice.

In media composed of a constant amount of fibrin and a mixture of Tyrodes solution and serum in varied concentrations, the amount of growth appeared to be independent of the concentration, and even of the presence of serum. According to Carrel and Ebeling¹⁰¹, neither serum nor fibrin is utilized by the cells. Carrel⁷³) found, that the rate of growth of fibroblasts grown in fibrin fixed in formaldehyde solution did not differ from that in normal fibrin. When fragments of the old strain of fibroblasts were cultivated in media containing a constant amount of serum and fibrin, and the concentration of embryo juice was varied, the rate of growth was found to be a function of the concentration of the embryonic juice in the medium.

And it has been shown by Carrel and Ebeling^{33) 34)} that the inhibiting influence of homo or heterogenic plasma varied in inverse ratio to the age of the animal from which the plasma was taken. The decrease in the rate of growth was 50 per cent during the first three years of life, whereas in the following six years it was only 30 per cent. Also the duration of life of the fibroblasts in vitro varied in inverse ratio to the age of the animal and decreased more rapidly than the age increased. From those experiments it was also concluded that during the course of life an increase of the inhibitory factor takes place, and not a loss of an accelerating one. It can therefore be emphasized that the substances which greatly accelerate the multiplication of fibroblasts are found in the tissue cells themselves, and do not exist in the blood serum.

Carrel 1912⁵⁶⁾ has shown that extracts from various tissues increased the rate of growth of fibroblasts very markedly. It was shown that extracts of embryos, of adult spleen and of Rous chicken sarcoma^{*)} had a marked increasing effect on the rate of multiplication of fibroblasts. Several years later Carrel and Ebeling proved that the embryonic tissue juice was able to carry on an indefinite multiplication of fibroblasts in vitro. In his early publication in 1913, „Artificial activation of growth of connective tissue“, Carrel⁵⁸⁾ showed that the activating power of the embryonic juice was reduced when heated to 56° C. and removed when heated at 70° C. By filtration through a Berkefeld filter the activation power was decreased markedly and completely suppressed by filtration through a Chamberland's filter. These simple treatments of the activating substances (tissue-juice) shows that the nature of those substances is very unstable.

As indicated by Carrel⁵⁸⁾ in his paper on the artificial

*) In a recent publication Carrel⁷³⁾ claims that the growth-promoting effect of the extract of Rous sarcoma is only apparent, and it is not able to promote the growth of fibroblasts indefinitely.

activation of tissue growth, it would be extremely interesting and even practically useful if such substances could be isolated. Firstly, the mechanism of growth could be understood and secondly, processes of repair could be accelerated. As Carrel⁵⁸ stated: "If the rate of the reparation of tissues were activated ten times only, a cutaneous wound would heal in less than twenty-four hours and a fracture of the leg would be cured in four or five days".

As the experiment of Carrel showed, that heating of the activating substances to 56° C. diminished the activating power and that even a filtration through a Berkefeld filter resulted in much less activating substances, I would imagine that the questionable substances would be difficult to isolate or separate on account of its lability. Although there was only a slight hope of getting hold of substances or portions of the tissue juice which had a still more activating power on the growth of tissues when separated from certain indifferent or growth-inhibiting substances in the tissue juice, it was worth while to undertake such experiments in order to get some ideas or suggestions as to the nature of those substances.

A few investigators have already tried to cultivate tissue cells in media with known composition. Lewis and Lewis^{337, 350} have used different salt solutions (seawater, Locke's solution and several modifications). As mentioned before, these inorganic solutions are not nourishing, but protective, and consequently cannot be used as culture media. O. Swezy⁴⁹⁰ cultivated fibroblasts in culture media consisting of egg albumin and Ringer's solution. It has not been possible to keep the tissue alive in those media for any noticeable length of time. Burrows and C. A. Neymann⁴⁰ added to the plasma medium carbohydrates, fats, peptones and α -amino acids. They did not observe any activation of the growth: on the contrary, it was observed that 2—5 per cent peptones prepared from the egg yolk had no effect and the addition of α -amino acids on the

contrary had a toxic effect on the tissue cells. In the experiments care was naturally taken to keep the culture medium isotonic.

The influence of extracts from various tissues, from embryonic as well as adult tissues, has been tried in the cultures (Carrel ⁵⁶, Wallon ⁵⁰⁷). As known, Carrel found that extracts of all different kinds of tissues activated the growth of fibroblasts. The degree of acceleration varied much. Embryonic tissue extract was the most active, and extracts of adult spleen and the Rous sarcoma were almost as active as the extract of chicken embryos. The effect of extracts of the thyroid gland and muscle of the dog on the growth of periosteum was very marked, but thyroid extract was more active than muscle extract. Carrel already observed before he worked with tissue cultures, that pulp of embryonic tissues placed in an open wound resulted in a rapid cicatritation. Also Wallon ⁵⁰⁷ tried the effect of extracts of various tissues on the growth of mammalian tissue in vitro. He found that most extracts stimulate the growth and that an extract of the liver has an inhibiting effect. Very recently Carrel ⁶³ has shown that an extract of leucocytes has a marked activating effect on the growth of fibroblasts and that leucocytes in culture are secreting substances which activate the growth of fibroblasts. (Trepheones), ⁶⁸).

PHYSICO-CHEMICAL STUDY OF THE NATURE OF THE GROWTH-PROMOTING SUBSTANCES.

To understand the mechanism of growth, it would be necessary to investigate the nature of the growth-promoting substances. If we, for instance, were able to isolate the factor which increases the velocity of cell multiplication, many hitherto unknown phenomena could be understood and processes of repair could probably be accelerated.

I began therefore to treat the embryonic tissue juice in different ways, to break it down by various chemical

operations to see if it was possible to concentrate its action by either evaporation to dryness and resolving it in small quantities of liquids — or by making fractions of the proteins in the tissue extract according to the generally known chemical principles. Most of these experiments gave, however, negative results, but they are anyway rather valuable because they contribute to characterize these substances and furnish a base for further experimentations.

The material I used for all the experiments, was tissue juice prepared from fresh chicken embryos. The tissue juice was prepared in the usual way as it designedly is done for the culture medium. Seven to ten days old chicken embryos were hashed up to a fine pulp with a pair of scissors; the pulp is centrifuged and the supernatant, clear, slightly mucous fluid is drawn off with a pipette. The fluid may simply be termed the "extract". At other times the extract was prepared in a slightly different way. To the embryos were added kieselgur and Ringer solution and they were ground in a mortar. It was found, that the extract obtained by the latter method possessed a much less activating power than did the extract obtained by cutting the embryos into small pieces with scissors. This fact agrees very well with the observation made on fresh embryonic extract, that it loses greatly its activity by merely shaking for some length of time. Carrel and Ebeling⁹⁵⁾ observed also that serum being shaken for some time lost a good deal of its activity. It is probable therefore that the grinding with sand or kieselgur effects the substances in a similar way as does the shaking. This shows, however, that the growth promoting substances must be of an extremely unstable nature. The first method was therefore selected for the preparation of the embryonic tissue juice. The old strain of fibroblasts¹⁶⁴⁾ (ten years old) was used as a reagent in the chemical experiments. Special techniques were developed for working aseptically during the different chemical and physical manipulations.

1. *Evaporation.*

The extract was placed in a thin layer in watch-glasses and evaporated to dryness. The evaporation was done in desiccators under vacuum and concentrated sulphuric acid or calcium chloride. It took usually from 3 to 5 hours until it was absolutely dried up.* It is, of course, important that the evaporation processes does not last long because the action of salt concentration may harm the delicate substances. The dry extract was dissolved in Ringer solution in different concentrations. Cultures with extract of the original, which had not been treated, served as controls for the cultures to which these extract solutions were added.

The result of these experiments was, that the growth-promoting substances had lost greatly in activity by the evaporation, even when the dry extract was dissolved in a smaller quantity of liquid than that in which it originally was dissolved.

2. *Precipitation experiments.*

Experiments were undertaken to isolate the different globulins and albumins in the tissue juice after the well-known chemical principles and measure the power of activation of the different fractions on cultures of fibroblasts.

Ammonium sulphate was used as a precipitant in a series of experiments. The extract was diluted equal to its own volume with Ringer solution and half saturated with $(\text{NH}_4)_2\text{SO}_4$. The solution was then centrifuged and the precipitates washed with distilled water until the ammonium sulphate was removed. The precipitates, which represented the globulins, were dissolved in a certain amount of Ringer solution. This solution was added to the culture medium and the rate of growth of fibroblasts determined. As controls served fibroblasts from the same fragments as used in the culture of the experiment, but

*) Quite recently, experiments of evaporating the embryo extract in a very quick way (a few seconds) did not give any better results.

cultivated in pure plasma without addition of any extract but diluted with Ringer solution to compensate the dilution of the experimental culture.

The cultures to which the globulin fraction was added, showed slightly more growth than did the control cultures in pure plasma. In other words, only a small portion of the growth promoting substances remained in the globulin fraction.

The albumins were obtained by saturation with Ammonium sulphate of the filtrate from the globulins and the ammonium sulphate removed by dialyzation. This fraction did not show any activating power at all. Similar results were obtained by Carrel for the serum albumins.

It is probable that the chemical procedures are rather rough and have a destroying effect on the growth promoting substances. It seems, however, that the active substances are to be found in the globulin fraction. This was also expected from the experiments made by Carrel and Ebeling¹⁰². They found, that the active substances of serum, could be detected in the globulin fraction.

It was important to find other methods of precipitation being less aggressive to the growth promoting substances. By using carbondioxide as a precipitant for the preparation of the globulins, we found a method more suitable.

3. Carbondioxide precipitation of the globulins.

The extract was diluted from four to twenty times with redistilled water. Kept at 0° C., CO₂ was allowed to trickle through the solution for about half an hour. The precipitate obtained after centrifuging was washed in water saturated with CO₂ at 0° C. After being washed that way, it was immediately after dissolved in Ringer solution and brought up to the original volume, and the hydrogen ion concentration adjusted to about Ph. 7. The filtrate from the globulins was evaporated at 40° C. to its original volume. This fraction contains the albumins.

The effect of the globulins and albumins obtained by precipitation with CO_2 proved to be slightly more active than the globulins obtained by precipitation with $(\text{NH}_4)_2\text{SO}_4$. From the table 1 such an experiment can be seen

Table 1.
Experiment No. 94.

	Culture No.	Absolute increase after 48 h.	$\frac{c}{c}$
Control	2243—1	5,17	0,5
Globulin	2243—2	2,6	
Control	2244—1	4,5	0,2
Albumin	2244—2	1,0	

To the control cultures were added the original untreated extract. The total nitrogen was determined of the original extract and of each of the two fractions containing the globulins and albumins. (Table 2)

Table 2.

Fraction	Per cent Total nitrogen.
Original extract	0,126
Globulin	0,047
Albumin	0,062

4. Alcohol precipitation.

Several other methods were used for precipitating the different proteins, but without any remarkable results. The method which gave the best results must be mentioned.

To the embryonic tissue juice was added 95 per cent alcohol until a precipitate appeared which was quickly removed by centrifuging, shaken with ether in order to remove the alcohol and resolved in Ringer solution. It is important to separate the precipitate quickly from the bulk. If not, the proteins will denaturate and be difficult to bring in

solution. Carrel,* who had the opportunity of trying these alcohol precipitates in the culture, stated that they increased the rate of growth nearly as much as the fresh extract, a fact to which I also subscribe.

Encouraged by these experiments, I attempted to develop a method by which it could perhaps be possible to fractionize different proteins by alcohol precipitation.

It is known, that the solution or colloidal system of proteins is very unstable close to the iso-electric point. If the hydrogen ion concentration of the solution of a protein is near that of the isoelectric point of the protein, the physico-chemical equilibrium is unstable and a very little disturbance of any kind is able to bring about a precipitation. It is known from Loeb³⁷¹ that at the iso-electric point the alcohol number is very low; i. e., a relatively small amount of 95 per cent alcohol is able to give a precipitation close to the isoelectric point of the protein. At the same time as the alcohol number is at its minimum, the conductivity, osmotic pressure and swelling also attain a minimum at the iso-electric point.

If we assume that the body juices, in this case the embryonic tissue juice and blood plasma, are built up of series of more or less definite proteins with different iso-electric points, we would expect, that at different hydrogen ion concentrations, we would be able to precipitate, by a certain amount of alcohol those of the proteins which are nearest the iso-electric point at the given Ph.

Experiments of this kind were made in the following way. Series of nine test tubes contained 5 c.c. each of freshly prepared embryonic tissue juice, 5 c.c. of standard buffer solutions with different Ph. were added, so that the extract-buffer mixtures all got a different hydrogen ion concentration, which varied from Ph. 5.6 to 7.5. To all of these tubes were added 95 per cent alcohol in quantities just

*) Personal communication.

sufficient to obtain a precipitate. This was removed quickly by centrifuging and washed twice with ether and dissolved in 5 c.c. of Ringer solution. The rate of growth of fibroblasts 10 years old strain ¹⁶⁴ was determined for the different fractions added to the culture medium. As controls served the original extract.

From table 3 it can be seen, that the higher the acidity is, the lower is the alcohol number. It can also be seen, that the transparency of the redissolved alcohol precipitate is highest at Ph. 6,8, at which reaction the precipitate was easiest to bring to solution. The effect of the different protein fractions on the rate of growth is such, that it increases markedly with the increasing alcohol number and decreasing hydrogen ion concentration up to a certain point, viz: to the normal reaction of the tissue juice at Ph. 6,8.

Table 3.

No.	C. C. Embryonic Tissue Juice.	Ph. of 5 c.c. Buffer solution added.	Ph. of Mix- ture of Tissue Juice and Buf- fer solution.	Alcohol Number in C. C.	Grade of Transparency of Re-Dissolved Precipitate.	Relative Increase of Growth of Fibro- blasts.
1	5,0	4,0	5,6	3,5	XXXX	0,07
2	5,0	4,6	5,8	5,5	XXXX	0,8
3	5,0	5,2	6,0	6,0	XXX	1,3
4	5,0	5,8	6,2	7,5	XXX	1,8
5	5,0	6,6	6,8	9,5	XX	0,9
6	5,0	7,0	6,8	11,0	X	2,4
7	5,0	7,8	7,2	11,5	XXXX	0,6
8	5,0	8,4	7,3	14,0	XXXX	0,8
9	5,0	9,0	7,5	16,0	XXXX	0,03

X — transparent, clear.

XXXX — highly intransparent.

This experiment does not seem, I believe, to indicate, that we by this method get precipitation of different proteins. If this should have been the case, we would not have got such great differences in the alcohol number and would have ob-

tained a precipitate by almost the same amount of alcohol in all the tubes. Plotted in a curve, the alcohol number of the extract under different hydrogen ion concentration gives almost a straight line, Fig. 2. The differences in the growth-indexes obtained may very well be explained as a result of the different solubilities of the acid- and alkali proteins produced, rather than it should be explained as the result

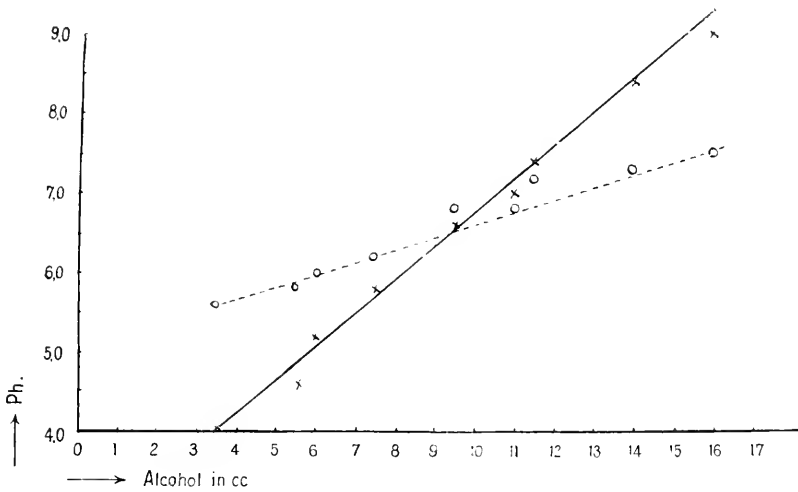


Fig. 2.

The solid line indicates the alcohol number of embryonic tissue juice under different pH. The dotted line indicates the pH of the redissolved precipitates.

of different more or less definite proteins. The experiment seems to speak much in favour of the assumption of Hertzfeld and Klinger^{256, 257}. They consider the proteins in the blood and body juices as a series of disperse systems with different sized particles, from the biggest represented in the fibrinogen and globulins and down to the smallest dialysable products, and not as a compound of more or less characteristic, well defined chemical bodies.

5. *Autolysis and hydrolysis of the proteins.*

Being neither encouraged by the experiments mentioned above nor by reading about the experiments made by Burrows and Neymann¹⁰ in which they tried the effect of amino acids on the growth of fibroblasts and found, that they had a toxic action. I tried, however, to hydrolyse the proteins of the embryonic tissue juice. I tried first to autolyse the tissue juice by keeping the tissue pulp and the juice in the incubator at 39° C. for different lengths of time, from a few hours to several days. The result of these experiments was, that the extract being exposed to the pulp at a temperature of 39° C. for a few hours lost greatly in activity. By digesting the tissue pulp with trypsin, no better results were obtained.

I tried then to hydrolyse the proteins of the embryonic tissue juice ad modum Kendall^{281, 282}, the method he used to isolate the thyroxin. By this method I obtained two fractions which Kendall called A and B. The fraction A was fatty and in consistency and colour like serum. This fraction could be brought into a kind of colloidal solution by boiling it with Ringer solution. The A fraction showed little or no effect on the fibroblasts; if any a toxic. The fraction B could easily be brought in a veritable solution. This solution gave no precipitate with acetic acid. Different concentrations of this B fraction were tried in the cultures of fibroblasts. Solutions were made which contained from 3.8 per cent and down to 0.05 per cent. A solution containing 0.17 grams per hundred c.c. Ringer solution, showed a slight activating power on the growth of fibroblasts. However, it did not have any toxic effect at all on the growth of fibroblasts as stated by Burrows and Neymann¹⁰. The amino nitrogen was determined in this fraction after the method of Van Slyke⁴⁷⁰ and found to be 1 per cent.

When it seemed that all these attempts to isolate a fraction which was supposed to have a growth promoting power

on the tissue cells, were without success, more indirect methods were selected with the purpose of studying the nature of this or these substances. A comparative quantitative analysis was therefore made of the amino acids in the hydrolysed embryonic tissue juice and the plasma from adult chickens, the latter containing none or very little of the growth promoting substances. It was supposed that some of the important amino acids were lacking or sparsely represented in the plasma or tissue juice. The results of these experiments were not very striking. In some of the analyses of the extract, lysine and histidine were found not to be present. In the plasma from the adult chickens all the amino acids sought for were there. It would be rather a interesting fact if the absence of these amino acids was constant in the hydrolysed proteins of the embryonic tissue juice.

Experiments No.s 85—87.

Table 4.

Quantitative determination of amino acids in hydrolyzed embryonic tissue juice and chicken plasma, a. m. Van Slyke.

		gram in 100 cc. of tissue juice	gram in 100 cc. of chicken plasma
Total N.		0,266	0,798
Amid N.		0,031	0,076
Melanin N.		0,0020	0,012
Total N. of the bases		0,032	0,210
Precipitated by phospho- tungstic acid. Basic amino acids.	Nonamino Nitrogen	Arginin	0,023
		Histidine	0,0
	Amino Nitrogen	Lysine	0,0
		Cystine	0,033 (?)
Total N. of the acid amino acids		0,164	0,460
1) Amino N. of the acid amino acids		0,14	0,360
2) Non amino N. of the acid groups			0,108

1) Contain: glutaminic acid, aspartic acid, Tyrosine, Phenylalanine, Serine. Leucine, Isoleucine, Valine, Alanine, Glycocoll.

2) Proline, Oxyproline, Tryptophane.

6. *Experiments with the fatty components of the extract.*

The effects of the fatty extracts of the tissue juice on the fibroblasts were absolutely negative; the same was found to be the case with regard to the tissue juice after the fatty substances had been removed. The fat extractions were done with the dry tissue juice in Soxhlet's apparatus. Petroleum ether was used. The tissue juice was extracted for 24 hours. After the evaporation of the ether the fatty substances were exposed to cold acetone. A part of it was dissolved and a part remained undissolved. Both of the acetone soluble and of the insoluble some kinds of suspensions in Ringer solution were prepared and added to the culture medium. Some of these substances were also saponified with NaOH and in this form added to the culture medium. All the substances had a toxic effect upon the tissue cells.

7. *Adsorption experiments.*

Experiments were undertaken to add indifferent substances as adsorbents to the embryonic tissue juice as adsorbendum to see if it was possible to adsorb substances which in one way or another were responsible for the growth promoting power of the juice – and afterwards to submit the adsorbed and unadsorbed substances to a chemical analysis.

Numerous experiments of this kind were undertaken. Different substances were used as adsorbent, such as animal charcoal, blood coal, mastic, gum arabic, barium sulphate, powdered agar and gelatine, stroma of tissue cells and fibrin. Of all of these the blood coal was most suitable and frequently used. The adsorption experiments showed often very different and rather inconstant results, probably due to the fact, that the tissue juices and plasma can not very well be standardized. They vary much, which can be seen, for instance, by the different amounts of totally nitrogenous substances they contain. In experiments of this kind the

rate of growth does not depend upon the presence of growth promoting substances only, but also upon the concentrations of various substances, the osmotic pressure, surface tension and many other physico-chemical factors. The technique and some of the experiments will be described in detail, because this method is found to be most preserving for the delicate substances, and will certainly contribute to the understanding of the nature of the growth promoting substances in the body juices.

Besides a number of preliminary experiments, where proper amount of adsorbent to be used was found, all the experiments were made under the control of the hydrogen ion concentration.

The recent investigations of Loeb³⁷¹) on amphoteric colloids has shown what an important and determining factor the hydrogen ion concentration is for the process of adsorption or more correctly, the chemical binding. As we have no idea of the chemical nature of the growth promoting substances, and we do not know the hydrogen ion concentration by which we shall obtain union with the adsorbent, it was therefore necessary to know a little about the character of the adsorbing materials. The different adsorbents were then investigated by means of cataphoresis experiments to find out their respective iso-electric points. According to the electrical charges of the dissociated protein bodies, the cationic and anionic aggregates will be adsorbed either on the one or the other side of the iso-electric point of the adsorbent; this depending only upon the hydrogen ion concentration of the solution.

Experiments were undertaken to see, at what hydrogen ion concentration the adsorption of the growth promoting substances has its maximum, and furthermore to see to what an extent these adsorbed substances could be displaced from the adsorbent. At the same time chemical analysis were made of the adsorbent including the adsorbed substances as well as of the supernatant fluid. The rate of growth was

determined for fibroblasts grown in the cultures to which media the different fractions were added and the growth-index compared with the amount of nitrogenous substances in the same fractions.

Table 5.

No.	Ph. of 1,5 cc. Buffer Solution.	CC. Fresh Embry Tissue Juice.	Gram Blood Coal.	Per Cent Nitrogen in Supernatant Fluid.	Absolute increase.
1	5,0	1,5	0,3	0,042	1,8
2	6,0	1,5	0,3	0,049	0,7
3	7,0	1,5	0,3	0,056	17,0
4	8,0	1,5	0,3	0,049	11,9
1	5,0	1,5	0,0	0,105	1,0
2	6,0	1,5	0,0	0,112	3,2
3	7,0	1,5	0,0	0,126	8,4
4	8,0	1,5	0,0	0,105	11,2

TECHNIQUE.

Mostly experiments of adsorption were made with blood coal as adsorbent. The concentration of the adsorbent used was 10 % and this was found most satisfactory to work with.

Fresh embryonic tissue juice from about 8 days old chicken embryos was prepared in the usual way. 1,5 cc. of this was carefully measured in series of test tubes. 1,5 cc. of a standard buffer solution of different Ph. were added to the juice in order to give the solutions certain different hydrogen ion concentrations. The method is the same as has been described before (Fischer¹⁹⁰⁵). After the buffer solution has been added and the desired Ph. obtained, 0,3 gram of blood coal was added. It is obvious, that the procedures were carried out strictly aseptically, with sterile solutions and glasswares. As control for the adsorption experiment another series of the same extract was mixed with exactly the same buffer solutions, but with the ex-

ception of the blood coal. Both series of test tubes, experiments and controls were placed in the incubator at 39° C. for one hour and shaken every 10 minutes. Hereafter they were placed in the refrigerator over night. The following morning the solutions were centrifuged, the supernatant fluid drawn off and the hydrogen ion concentration adjusted for all of them to Ph. 7.0, by adding various amounts of $n/10$ HCl or NaOH, an amount, which was found empirically. After this neutralization, the solutions were ready to add to the culture media and the rate of growth determined. The tissue used as a reagent was the then 9 years old strain of fibroblasts in the *Rockefeller Institute*. As usual only good cultures were used for the experiments. The tissue fragment was cut into two halves, the one served as control and the other for the experiment. The control contained one volume of plasma and one volume of the tissue juice-buffer mixture which previously was neutralized to Ph. 7.0, i. e. having been treated in the same way as the experimental extract, with the exception of the adsorbent. The experimental culture contained one volume of the supernatant fluid from the blood coal, after of course also having been neutralized to Ph. 7.0, and one volume of plasma. The method for quantitative estimation of the rate of growth was the usual as often before described. ¹⁶³).

It has been endeavoured to see if it was possible to remove a part of the adsorbed substances from the adsorbent, and especially in the cases where a marked adsorption of active substances has taken place.

It has been shown, that antibodies which had once combined with adsorbent or homologous antigen, can be removed from their attachment and rendered reavailable. F. HUNTOON ²⁶⁷⁻²⁶⁸) has studied the conditions under which the dissociation of antibodies takes place. He declares, that antibodies are bound to the antigen in all different grades of stability, from that part which is very loosely bound and easily washed out. He found, that the dissociation was very

often best when he used water without salts or else water with salts in excess.

I tried similar arrangements as Hunt²⁶⁷⁻²⁶⁸. Fresh embryonic tissue juice was mixed with 10 per cent blood coal and a buffer solution which gave the mixture such a hydrogen ion concentration by which we empirically found that a maximal adsorption of growth promoting substances took place. After one hour at 39° C. and 12 hours in the refrigerator, the blood coal was separated from the fluid by centrifuging. After the supernatant fluid had been removed, various solutions such as distilled water, Ringer solution, 10 per cent saccharose solution, were added to the different tubes with blood coal. The coal was shaken up and some of the tubes were placed at 53° C. for two hours, some at 39° C. and some directly placed in the refrigerator, where they all were kept for about 15 hours. At the end of this time, the supernatant fluid was drawn off and added to the cultures and the rate of growth determined. The adsorption was controlled chemically by also determining the amounts of nitrogenous substances adsorbed and dissociated by the various methods.

It is generally known, that highly surface active substances can displace certain adsorbed substances from the adsorbent. Rona and Tosh¹⁵⁴ found that ethyl-urethane was able to displace the adsorbed substances. An attempt with ethyl-urethane was therefore made to displace the adsorbed growth promoting substances. Some preliminary experiments were made to investigate the displacing power of various surface active substances. At the same time these substances were tried in the cultures in the concentrations where a good displacing effect was obtained.

In these preliminary experiments on displacement by surface active substances, blood coal was used as adsorbent and a 0.3 per cent solution of methylene blue in water as adsorbendum. After the adsorption had taken place, the solution was separated from the blood coal and the latter washed

until the water remained colourless. Water was then added until the original volume as before was established. The displacing agent was then added to the tubes in different concentrations and the blood coal stirred up. The colour of the water could then be measured colourimetrically at the same time as the surface tension could be determined. The experiment in table 6 shows that the lowering of the surface tension about 8–9 dynes results in a displacement of methylene blue from the adsorbent, when 95 per cent alcohol is used as displacing agent.

Table 6.

Showing the effect of some surface active substances on adsorbed methylene blue.

No.	Gram blood coal.	cc. Water.	cc 95 $\frac{0}{10}$ alcohol.	cc. $\frac{1}{2}$ $\frac{0}{10}$ Na oleate.	Colour.	Surf. ten. in dynes.
1	0,3	3,0	0,03	0,0	clear	118
2	0,3	3,0	0,08	0,0	slight blue	111
3	0,3	3,0	0,14	0,0	intense blue	106
4	0,3	3,0	0,0	1,0	dark blue	51
5	0,0	3,0	0,0	0,0	dark blue	120

Of course it cannot be concluded from this experiment, that other substances, as for instance the growth promoting substances from the embryonic tissue juice, can be displaced from the adsorbent in the same way by lowering the surface tension to the same degree which was able to displace the methylene blue. This depends upon several factors, the solubility of the adsorbed substances in the displacing agent and the surface energy of the adsorbed substances in relation to the surface energy of the displacing agent. Ethyl-urethane was able only to lower the surface tension of Ringer solution, when present in a rather high concentration.

The concentrations of sodium oleate and ethyl-urethane by which they were able to displace methylene blue from

the adsorbent was found to have no toxic effect on the growth of fibroblasts.

RESULTS.

Two sets of test tubes, 4 in each set were used in an experiment. The one set had blood coal as adsorbent and the other had no adsorbent. All the tubes contained 1.5 cc. of freshly prepared embryonic tissue juice and 1.5 cc. of a buffer solution which gave the solutions different hydrogen ion concentration. The 4 tubes with the adsorbent have exactly the same Ph. as the 4 tubes without the adsorbent. To the number one of each set is added a buffer solution with Ph. 5.0 and to the number two of each set is added a buffer solution with Ph. 6.0 and so on. The total nitrogen was determined of the supernatant fluid in the set which contained blood coal and of the fluid in the set which did not contain the adsorbent. After the hydrogen ion concentration was adjusted to Ph. 7.0, the fluids were added to the culture media and the rate of growth for the fibroblasts determined.

It was found that the total nitrogen in the supernatant fluid was reduced to about 40—50 per cent when 10 per cent blood coal was used as adsorbent, whereas the growth promoting activity of the supernatant fluid differed rather much in the various experiments and was not constant at all. It can be summarized in stating, that the growth promoting power was least when the adsorption took place at a relative high hydrogen ion concentration; in spite of the after neutralization there remained a permanent change in this portion, namely a less activating power as compared with that of the other solutions. This permanent change towards a less active power, might be due to the effect of the hydrogen ion concentration and not due to any adsorption, because the same change was observed on the corresponding tube in which no adsorbent was present. Any visible precipitation could not be observed in the fluid without adsorbent.

Several times it was observed that the supernatant fluid from the tube containing extract and adsorbent at the neutral point, showed more activity than the original fresh concentrated extract. This may either be due to a relatively extensive adsorption of growth-inhibiting substances or to the dilution of the extract. Generally it was observed that the supernatant fluid was less active than the original native solution, on either side of the neutral point.

By determining the nitrogen in the supernatant fluid, it was observed, that from 50 to 60 per cent of the total nitrogen of the extract was adsorbed — furthermore it was found, that about 30 per cent of the adsorbed nitrogenous substances could be dissociated from the adsorbent. This was rather constant, no matter which method was used for the dissociation. The growth promoting power of the displaced substances was very different in the different methods. The best way in getting a good dissociation of growth promoting substances was to add Ringer solution with 0.2 c.c. of a 1 per cent sodium oleate and keep it for about two hours at 39° C. and 15 hours in the refrigerator. None of the dissociated solutions showed approximately as activating a power as the original extract. Absolute destruction of the activity of the extract was the result of the method of dissociation at 53° C. for two hours.

The amount of nitrogenous substances which could be dissociated was the largest when the adsorption had taken place at Ph. 7.0 in spite of the fact, that the amount of adsorbed was found to be the least at this reaction.

The amino acids remained in the bulk and were not adsorbed to any measurable extent. The determination of the amino acids was made after the method of Sørensen by the formol titration. It is interesting enough to see that the amino acids were not adsorbed in spite of the fact that 50 per cent of the nitrogenous substances were adsorbed. It is very probable, that it is the largest molecules that are responsible for the activity. The experiments of ultra fil-

itation made by Carrel*) speaks very much in favour of this assumption.

The table 7 represents an experiment of adsorption, followed by the dissociation by means of different methods. The adsorption and dissociation were all the time controlled by nitrogen determinations of the various fractions.

Table 7.
Adsorption and dissociation experiment.

No.	Grams of blood coal.	cc. of fresh extract.	Ph. 5,6 cc. of buffersol.	Per Cent N in supernat. fluid.
1	0,3	1,5	1,5	0,042
2	0,3	1,5	1,5	0,042
3	0,3	1,5	1,5	0,063
4	0,3	1,5	1,5	
5	0,3	1,5	1,5	0,056
6	0,0	1,5	1,5	0,105

The supernatant fluids were then drawn off and the dissociating fluids were added to the blood coal and shaken. The different tubes were treated in the following ways.

- 1 3 c.c. of distilled water; two hours at 53° C.; 15 hours in refrigerator.
- 2 3 c.c. of destill. water; two hours at 39° C.; 15 hours in refrigerator.
- 3 3 c.c. of 10 % saccharose sol. at 39° C. for two hours; dialyzation and the volume adjusted.
- 4 3 c.c. of Ringer solut.; two hours at 39° C.; 15 hours in refrigerator.
- 5 3 c.c. of Ringer solut. containing 0.2 c.c. of a 1 % solution of sodium oleate; two hours at 39° C.; 15 hours in refrigerator.
- 6 Two hours at 39° C. and 15 hours in the refrigerator.

*) Unpublished experiments.

The nitrogen was then determined in the blood coal and in the supernatant fluid separately after the dissociation has taken place. The absolute increases of growth of fibroblasts were determined in the various fractions. Table 8.

Table 8.

No.	Per Cent N in supernat. fluid.	Per Cent N in blood coal.	The absolute increase of growth in sq. cm.
1	0,021	0,079	0,75
2	0,021	0,075	2,8
3	0,021	0,075	spoiled
4	0,021	0,075	1,64
5	0,021	0,075	4,1

The undiluted original extract for these experiments contained 0,21 % total nitrogen -- and the absolute increase of growth was found to be 12,7.

When diluted with equal its volume of buffer solution Ph. 5,6, the total nitrogen was found to be 0,105 -- and the absolute increase was 6,2.

The supernatant fluid from the adsorption experiments showed a positive Heller, Millon and Biuret reaction, but the dissociated substances did not give these reactions. The following experiment illustrates this Table 9.

Table 9.

	Total nitrogen.	Formol titr. cc. n/10 NAOH per 100 cc.	Heller	Millon	Biuret	Abs. increase of growth.
Original undilut. extr.	0,133	10	+	+	+	22,8
Supernat. fluid f. adsorb.	0,091	10	+	+	+	10,8
Dissociated extract.	0,028	—	÷	÷	÷	4,3

These adsorption experiments will probably pave the way for the investigation of such complex substances. The interpretation of the experiments is of course difficult be-

cause of the rather inconstant results obtained here, but doubtlessly experimental arrangements similar to those mentioned here will be more useful than the mere chemical analysis which at the present time is too aggressive for the delicate growth promoting substances.

SUMMARY AND DISCUSSION.

The culture medium for cultivation of tissue cells in vitro is apprehended in two parts namely 1) the supporting apparatus and 2 the growth promoting substances.

The supporting apparatus of the plasma culture is represented in the fibrin. This can be substituted by certain other indifferent substances such as spider-web, glass-wool, silk- and cotton threads and almost any indifferent material. It is important to know, that there exists a certain relation between the amounts of fluid and supporting material. When the liquid phase is held by capillary suction to the framework the best conditions are established for an outgrowth of the cells.

The second component of the culture medium is essential for the indefinite growth of tissue cells in vitro, namely the growth promoting substances. These are not found to be present in the plasma from young or old animals in any sufficient quantity to keep the cells living permanently outside the body. Whereas they are found to be present in the tissue cells themselves, preferably in certain tissues such as embryonic tissue, spleen, liver, leucocytes from adult animals and in certain sarcoma. Here it must, naturally, be understood, that we are only dealing with the cultivation of tissue cells in homologous media, i. e. media derived from the same species of animal as the tissue cells. The cultivation of cells in heterologous media is entirely another matter and the investigation on this field is rather new and insufficiently worked out to permit any conclusions being drawn.

A fragment of tissue explanted in a protective solution Ringer, Lockes or Locke-Lewis is able to live for

a short length of time at the expense of the growth promoting substances contained in the tissue fragment itself, and cannot therefore be considered as a real tissue culture in *sensu strictiori*, but a mere survival.

Experiments were undertaken to isolate a fraction of the embryonic tissue juice, which contains the actual growth promoting substances. The results were rather negative as it proved to be impossible by employing the ordinary known chemical methods for breaking down the proteins to simpler fractions. The results are therefore more valuable from the point of view, that they have contributed to characterize the nature of these substances. The experiments made here cannot at all, therefore, be considered as completed but each of them is a problem in itself and need further investigation. Considering that these experiments are the first of their kind, they merely indicate the line of work and contribute to the making of the first rough characterization of the substances.

The conclusions of the chemical experiments may be summarized thus: The growth promoting substances are exceedingly unstable. They are suppressed at 37° C. for a few hours and completely inactivated at 56° C. for one hour. Shaking and filtration through Berkefeld and Chamberlands filters also suppress their activity.

Different methods were applied for preparing fractions of the protein solutions. Precipitation of the globulins and albumins was carried out by using salts of the heavy metals, carbon-dioxide and by dialyzation. These processes caused practically an inactivation of the growth promoting substances, though it can be concluded that the active substances are precipitated with the globulins. The albumins did not show any activating power at all. The CO₂ method for precipitating the globulins proved to be the most conservative for the labile substances.

Precipitation of the proteins with 95 % alcohol, followed by a quick washing with ether, proved to be the best method



for obtaining the growth promoting substances. The substances obtained by this method were almost as active as the native solution.

The amino-acids from the hydrolyzed embryonic tissue juice showed no toxic effect on the fibroblasts in vitro, as has been stated for amino-acids in general by Burrows and Neymann ¹⁰⁾.

Various indirect methods were selected, so as to endeavour to obtain some information as to the classification of the active substances.

The embryonic tissue juice as well as the adult plasma was hydrolyzed and the amino-acids determined quantitatively ad modum Van Slyke ¹⁶⁹⁾, so as to make a qualitative as well as a quantitative comparison between the amino-acids in the blood-plasma which have no activating effect at all, and the embryonic tissue juice which contains the growth promoting substances. The results were rather inconstant and not clear.

Different adsorption experiments were undertaken, in order to find new and more conservative methods for the study of these substances. Blood coal was generally used as adsorbent in a concentration of about 10 %. The adsorption was investigated under influence of different hydrogen ion concentrations of the tissue juice. It was observed that at Ph. 7.0 the supernatant fluid from the adsorbent blood coal possessed almost all the activating power of the tissue juice, in spite of the fact that almost 50 % of the total nitrogen of the solution was adsorbed. At both sides of the neutral point, the supernatant fluid, when brought back to Ph. 7.0 after the adsorption, remained inactive.

The substances adsorbed are those belonging to the biggest molecules. The dialysable substances, the amino-acids, remained unadsorbed at the different hydrogen ion concentrations.

In other experiments it was tried to displace the substances once adsorbed by washing the adsorbent with va-

rious solutions containing surface active substances. About 30 % of the total nitrogen adsorbed could be dissociated from the blood coal. The displaced substances did not seem to possess any striking effect on the cell-activity.

The fact that direct chemical tests are not yet available to determine the chemical nature of the so-called growth promoting substances, leads us to find other indirect methods of studying them.

As indicated before, I got an impression that it might probably be explained that individual definite protein or substances do not exist, which alone are responsible for the growth promoting power, but the activity of the tissue juice might very well be the result of physico-chemical interactions in the colloidal system. The proteins in the body juices represent a polydispersoid system and its physical properties, surface energy, viscosity, electrical charge and osmotic pressure, are very much influenced by the active reacting substances, which so far have been used for the separation of the supposed more or less definite compounds.

We know now as facts, that the embryonic tissue juice freshly prepared, has the ability to maintain life of tissue cells indefinitely outside the organism ^{58, 164}). We know also from the experiments that merely by storing the tissue juice for some time, even in the cold, it loses much of its growth promoting power; it is likewise known, that shaking ⁹⁵ the tissue juice or heating it to 56° C. greatly suppresses its power. These facts could very well be explained by the assumption that a change in the colloidal system takes place of a more physical character. Colloidal solutions differ from the true solutions only in the sizes of particles or molecules. The protein solutions are probably true solutions, but their molecules are so large that their physical properties very closely approach those of the colloids. We know that colloidal solutions of the simplest kind as ordinary metal sols for instance, are rather unstable in function of time and still more in function of temperature. Changes are

going on in the disperse phase, i. e. there is a tendency to decrease the relative surface of the disperse phase which results in a denaturation, a transition to a gel. The denaturation takes place without the presence of living cells and the rate of velocity of denaturation depends upon several factors such as the concentration, the character of the disperse medium, the electrolytes and temperature.

In the colloidal system, as we will consider the body juices, the surface forces play an enormously important rôle by changing the character. In vital processes the surface forces are by far the most important. Our experiments have taught us, that the tissue cells succumb very rapidly if they themselves are not allowed to spread out in between the solid and liquid phase. It was noticed how important the framework was for the life of tissue cells and how important the relationship was of the amount of liquid to the solids. Fischer.* On the surfaces of solids, cell membranes, colloidal particles, we have the theatre for the play of the physical forces, surface tension, potential differences, membran electricity and so on. Phenomena of oxidation usually occur at the free surfaces. The recent experiments of P. du Noüy⁴²², seem to elucidate these problems. The colloidal solutions seem to exhibit remarkable properties at the surface. Perhaps many of the phenomena can be explained by Donnan's and Langmuir⁵¹⁹ assumption of the molecular orientation on the free surfaces. We know after all that spontaneous changes take place in colloidal solutions. Changes of a similar kind might very well be responsible for the spontaneous inactivation of the growth promoting substances of the embryonic tissue juice.

As already mentioned, the body juices can be considered as a polydispersoid system in a kind of stationary condition in the body. This stationary condition is the result of the life processes in the organism. If any attempt is made to dis-

*) Unpublished experiments.

order this condition, a rapid recovery takes place. It is known how difficult it is to bring about a change, naturally within certain limits, in the osmotic pressure, hydrogen ion concentration in the juice of the intact organism. The disturbance has to attain a certain order of magnitude before the condition is altered and it takes then only a relatively short time to restore the balance. The tissue cells are responsible for the stationary condition in the body: the catabolic substances are eliminated, and the changes caused by the interactions of the colloidal systems i. e. the tendency to decrease the relative surface of the disperse phase, are prevented and do not come to a true physico-chemical terminal-equilibrium. The interval between this terminal-equilibrium and the stationary condition within the organism, might be called the kinetic of the juices. Perhaps the kinetics of the embryonic tissue juice have a much wider range than the body juices of the adult.

When we now consider the life of the tissues in vitro and make a comparison with that of the tissue cells in vivo, we have no elimination of the end-products in the culture. When we allow the cells to grow in the same culture medium for a long time, accumulation of catabolic substances causes the death of the cells after a certain length of time, when the body juices from the adult alone represent the culture medium. By adding tissue juice from the embryo to the culture medium the cells may be rendered able to live much longer in the same medium before they die, and the cause for this difference may be the different range of kinetics in the embryonic tissue juice and the plasma from the adult. In other words, the buffer action, if we may call it so, of the embryonic tissue juice is far greater than that of the adults and probably it is in the buffer action or kinetic range, that we may find the cause of the growth promoting power.

That life processess are influenced and dependant upon physico-chemical forces is beautifully demonstrated by J.

Loeb³⁶⁷, who showed that the duration of life of sea-urchin eggs, fertilized and unfertilized, are influenced by the temperature. Still better are the experiments of Loeb and Northrop³⁶⁸ with the fruit fly *Drosophila*, the life of which as function of temperature runs as a monomolecular reaction. Within a certain limit of temperature, the temperature coefficient is 2, i. e., that at an increase in temperature of 10° C. the duration of life is only half as long.

It can be seen, that the mechanistic apprehensions of the various life processes are the conclusions of clean cut experiments, which show the ultimate continuity between the living matter and the non living world.

Even if it, at the present time, looks as if growth promoting substances for the tissue cells cannot be found as definite substances, we are not allowed to give up the hope, that such substances exist. To day, we are working with many different substances of which we have no knowledge whatever. So much has been accomplished in immunology, in spite of the fact, that the reacting substances are not yet known or isolated. Also the vitamins and the hormones we do not know at all in pure condition.

Not many years ago it was believed that a solution of sodium chloride was a physiological solution, as soon as it was isotonic. To day we know that a isotonic sodium chloride solution is not sufficient to protect living cells. J. Loeb³⁶⁹ has shown, that a pure solution of sodium chloride is toxic for the cells and that this toxicity can be neutralized by bivalent or still better by trivalent ions, such as Ca^{++} and Mg^{+++} when added to the NaCl solution in a proper quantity, and that also K^+ is important for maintaining life. Loeb explained this phenomenon by stating that Na, K and Ca ions are combined with certain colloidal substances within the cells. These combinations in certain proportions in the cells are necessary for the normal vital processes. If one places the cells in pure NaCl solutions,

as a result of the mass action, the Na ions later substitute the K and Ca ions in the cell body and they die. In other words, NaCl is essential for life, nevertheless NaCl is toxic for the cells when it is present as the only salt. The analogy, that the growth promoting substances and growth inhibiting substances if such exist should act in some similar manner does not seem to be too hazardous a conclusion.

The work to be done in this line, is to find some indirect methods for investigating the nature of these substances, methods which do not interfere too much with the physical orientation of the colloidal or big-molecular solutions. The methods which are thought of here will probably tend in the same direction as used in the investigation of the immune bodies. The conditions are very complex and will require much patience and ingenious observation for their elucidation.

GROWTH OF FIBROBLASTS AND HYDROGEN ION CONCENTRATION OF THE MEDIUM.

In connection with the investigation of the physico-chemical properties of the growth promoting substances, experiments were undertaken to determine the rôle played by the hydrogen ion concentration of the culture medium in regard to the growth of fibroblasts cultivated *in vitro* for a long period of time.

The method used for the determination of the hydrogen ion concentration in small amounts of fluid was that of Felton¹⁸⁸⁵. For the experiments fibroblasts from a 1—2 months old strain and fibroblasts from the old strain of Carrels 9 years old were used.

The fibroblasts were cultivated for long periods of time in media of known hydrogen ion concentration. The variation in the hydrogen ion concentration of these media were obtained in the following ways.

Solutions of hydrochloric acid were prepared in such concentrations that the addition of one drop to a certain

amount of embryonic tissue juice or plasma would give the hydrogen ion concentration desired.

Or another method was this, that Sørensen's standard phosphate solutions, sterilized in pressure bottles, were added to the embryonic tissue juice in equal volumes to obtain different hydrogen ion concentrations of the media. It was observed that mixture of embryonic tissue juice and these standard phosphate solutions had no inhibiting effect

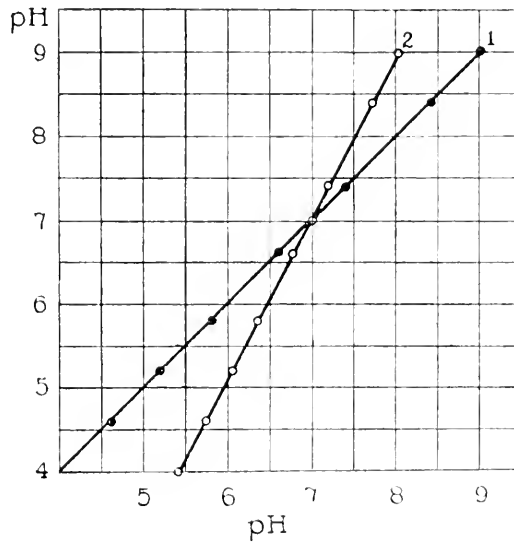


Fig. 3.

The abscissæ indicate the pH obtained, and the ordinates the pH of the buffer solution added. Curve 1 represents the pH of Ringer solution, and Curve 2 the pH of embryonic tissue juice, to both of which are added the different standardized buffers as indicated by the ordinates.

on the growth of fibroblasts except when the hydrogen ion concentration was such that this alone caused an inhibiting influence on the growth of the tissue. A known hydrogen ion concentration was obtained by the following technique. Preliminary experiments were made in order to find out the Ph of a mixture of embryonic tissue juice

and a given acid. Different numbers of drops of the respective solutions acid, alkali or buffer were added to tubes holding the same amounts of juice, and the hydrogen ion concentration was tested. These different known reactions of the juice gave a curve in which the amount of acid necessary to obtain a certain hydrogen ion concentration

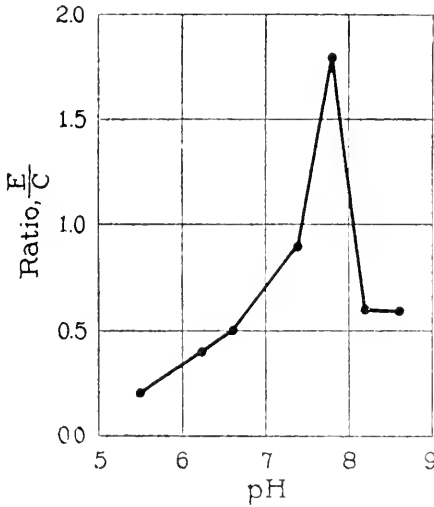


Fig. 4.

The different hydrogen ion concentrations of the media in which the fourth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth, $\frac{E}{C}$. The different hydrogen ion concentrations in the medium were obtained by adding phosphate buffer solutions to the extract.

between the points found empirically could be calculated by interpolation, (Fig. 3).

Equal volumes of the standardized buffer solutions and of the tissue juice were used. To know what Ph resulted in the tissue juice when it was mixed with its own volume of buffer solution, the reactions obtained gave a straight line when a curve was plotted, and it was possible to cal-

culate readily which buffer should be used to produce the hydrogen ion concentration desired in the tissue juice (fig. 3).

Hereafter it was necessary to make another test in the same way, namely with the plasma-tissue juice mixture. This had to be done rapidly in order to mix indicator and juice-plasma before coagulation took place. In other words, when fibroblasts are to be cultivated in a medium

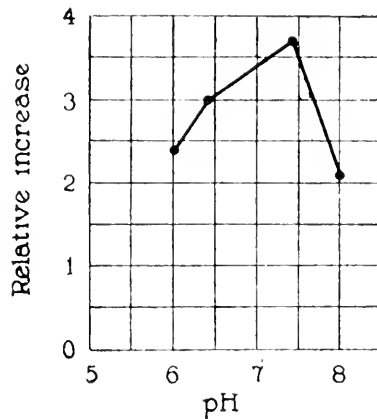


Fig. 5.

The different hydrogen ion concentrations of the media in which the first passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

of, let us say, Ph 6, preliminary experiments for those particular media will show what buffer solutions should be added to the juice which will give Ph 6, when combined with plasma. The test should be made in two steps for each experiment, one for the juice-buffer, and the other for the juice-buffer-plasma mixture, because of the slight differences in hydrogen ion concentration of the different juices and plasmas. The embryonic tissue juice may vary from Ph 6.8 to 7.2, and the plasma from Ph 7.1 to 8.0.

The culture of fibroblasts destined for the experiment was divided into two equal parts. One fragment was cultivated in a medium composed of 1 drop of plasma and 1 drop of a mixture of embryonic tissue juice and buffer

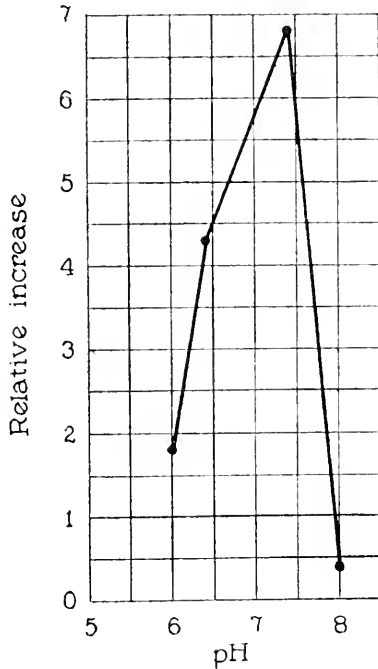


Fig. 6.

The different hydrogen ion concentrations of the media in which the fifth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

solution. The other fragment, the control, was cultivated in 1 drop of plasma and 1 drop of embryonic tissue juice to which previously had been added its own volume of Ringer's solution to compensate for the addition to the experimental culture of buffer solution. Mica cover-slip were used, because of the cover glass giving off alkali to the

medium. About one hour after the preparation of the culture, an outline drawing was traced under the projectoscope. After 48 hours incubation the outline of the new growth was drawn of the control and the experimental culture, and calculations were made by the method described by *W. J. P. M.*

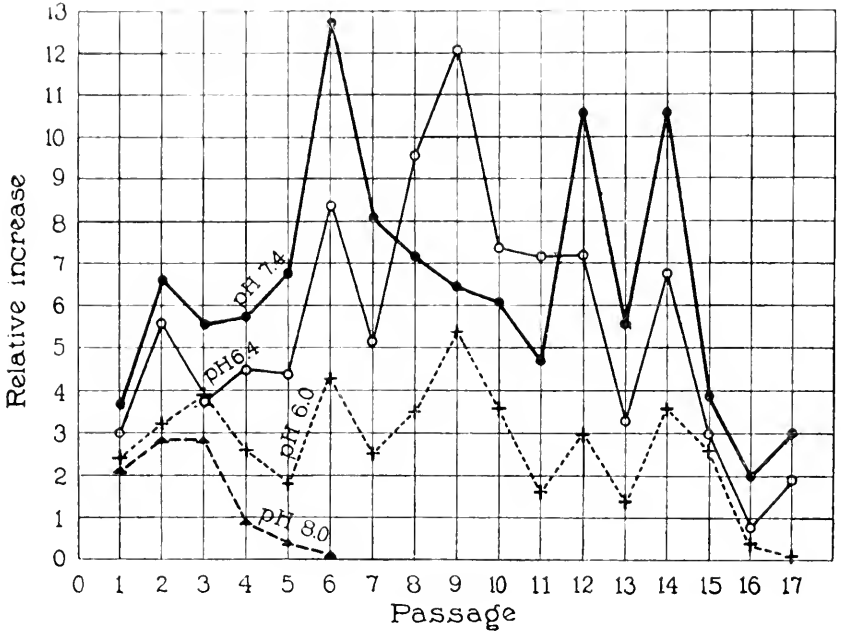


Fig. 7.
The growth of the four cultures of the experiments expressed graphically.

scribed in the beginning of this chapter. At each passage the culture was divided into two parts. One fragment was kept as control and the other placed in the experimental medium, care being taken to keep the other factors constant, such as the osmotic pressure and so on.

The curve obtained for the rate of growth of fibroblasts cultivated in media containing different hydrogen ion

concentrations varying from Ph 5.5 to 8.5, showed a distinct maximum of growth between Ph 7.1 and 7.8 figs. 1 to 6. The rate of growth decreased rapidly with increasing hydrogen ion concentration and hydroxyl ion concentration and the curve obtained was nearly symmetrical. The curves reached a maximum at Ph 7.1 and fell very rapidly on both sides of this point always a little steeper on the alkaline side in spite of the fact that the resistance against the alkalinity was more marked. The type of curves were the same in all experiments. By this method it was possible only to investigate a short range of concentrations of the hydrogen ion, because the coagulation process of the plasma was interfered with when the Ph was below 5.5 and above 8.5; sometimes precipitations occurred — so it proved impossible to draw any conclusions from the experiments beyond the said range.

If the series of experiments was repeated by carrying the fibroblasts into a new medium containing the same hydrogen ion concentration as used in the former culture medium, it was observed that the absolute increase of new growth became less and less in the most alkaline and most acid media, (fig. 7 and tables 10–11). The descent of the curve on both sides of the maximum became more abrupt in the succeeding generations because of the retardation of growth in the media with the highest alkalinity and acidity; compare fig. 5 which represents the first passage and fig. 6 which represents the fifth passage.

By following the growth of fibroblasts passage after passage, in media containing the same hydrogen ion concentration, it was observed that in the highest acidity pH 5.5 growth ceased after four to six passages. The fibroblasts showed more resistance to higher alkalinity, but at the highest grade of alkalinity pH 8.5 they grew for about eight to ten passages. — The optimum growth occurred between pH 7.0 and 7.8. This was found also to be the normal reaction of a mixture of plasma and embryonic

Table 10.

Experiment No.	Culture No.	Buffer solution added. *)		First passage.			Second passage.			Third passage.			Fourth passage.		
		Juice-buffer mixture.		Control	Experiment	E C	Control	Experiment	E C	Control	Experiment	E C	Control	Experiment	E C
		pH	pH												
1	264	4.0	5.5	7.0	0.4	0.6	6.2	3.1	0.5	3.0	2.7	0.9	6.1	1.7	0.2
2	265	4.6	6.0	5.4	0.1	0.1	6.3	2.4	0.3	6.6	3.0	0.4	6.5	0.9	0.1
3	266	5.2	6.2	3.1	7.3	0.4	16.5	4.7	0.2	3.0	1.7	0.5	6.6	3.0	0.4
4	267	5.8	6.6	1.8	1.5	0.7	8.7	4.1	0.4	4.6	3.3	0.7	3.0	1.7	0.5
5	268	6.6	7.2	4.7	3.9	0.8	12.4	1.8	0.1	4.2	5.1	1.2	5.8	3.4	0.5
6	269	7.0	7.4	10.8	10.8	1.0	12.2	11.9	0.9	4.8	6.8	1.4	7.3	6.6	0.9
7	270	7.4	7.8	10.4	6.6	0.6	4.3	5.1	1.1	9.9	12.1	1.2	2.1	3.9	1.8
8	271	8.4	8.2	11.8	4.2	0.3	12.1	7.9	0.6	10.3	8.1	0.7	3.7	2.3	0.6
9	272	9.0	8.6	7.2	6.3	0.8	11.1	7.7	0.7	12.7	8.4	0.6	7.6	4.9	0.6

Table 11.

Experiment No.	Culture No.	Buffer solution added. *)		Relative increase.																
		Juice-plasma-mixture.		Passage No.																
		pH	pH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	375-1	4.0	6.0	2.4	3.2	3.9	2.6	1.8	4.3	2.5	3.5	5.4	3.6	1.6	3.0	1.4	3.6	2.6	0.4	0
2	375-2	5.8	6.4	3.0	5.6	3.7	4.5	4.3	8.3	5.2	9.6	12.1	7.3	7.2	7.2	3.3	6.8	3.0	0.8	1.9
3	375-3	7.0	7.4	3.7	6.6	5.6	5.8	6.8	12.7	8.1	7.2	6.5	6.1	4.7	10.6	5.6	10.6	3.9	2.0	3.0
4	375-4	9.0	8.0	2.1	2.8	2.8	0.9	0.4	0	0	0	0	0	0	0	0	0	0	0	0

*) 1 cc. of buffer solution was added.

tissue juice. It can also be seen that the slightest variation of the hydrogen ion concentration of the culture medium resulted in marked changes in the rate of growth of fibroblasts. And it is interesting to notice here the fact, which has been mentioned before, that the differences were mostly of quantitative nature. The only morphological change of the cells observed was that, in the acid media they showed more vacuoles than in the alkaline.

Lewis and Felton³⁴⁸ have investigated the influence of the hydrogen ion concentration on the growth of fibroblasts in Locke-Lewis salt solution. They were able to investigate a much wider range of different hydrogen ion concentrations than I was able to do, on account of the liquid media they used for the experiments; on the other hand they were not able by their technique to investigate the prolonged action of various concentrations of the hydrogen ions on the tissue cells as I was able to do by the technique described here. They varied the hydrogen ion concentration from pH 4.0 to pH 9.2. They determined the hydrogen ion concentration by the method of Felton¹⁸⁸⁴ of the culture media at different stages of the growth of the cells. Fibroblasts cultivated in a medium with a hydrogen ion concentration of pH 4.0 to 5.5 seldom showed any growth; those in a medium of pH 5.5 exhibited growth in a few instances; while those in media having a hydrogen ion concentration from pH 6.0 to pH 9.0 usually showed abundant growth.

IV.

TECHNIQUE FOR TISSUE CULTIVATION.

The outlines of the method for cultivating tissue cells in vitro are very much the same to-day as described by Harrison ²¹⁶ in 1907. Before that time many investigators had already thought of the possibility of growing tissues outside the organism, but nothing really came of it. Harrison ²¹⁷ demonstrated that embryonic tissue of the frog, transplanted into coagulable lymph, was able to develop in a normal way. At that time Carrel was interested in cicatrization of wounds and decided to develop a technique which could be applied to the cultivation of adult tissues. Burrows studied the technique in Harrison's laboratory and together with Carrel they developed the beautiful technique for tissue cultivation which gradually has been modified and improved. But in the headlines the technique is the same to-day.

Instead of using the lymph as Harrison did, Burrows and Carrel used plasma from the same or from different species. It was soon found by Carrel and Burrows in what direction this line of research should go. It was observed that the tissue could be kept alive and actively growing by making secondary and tertiary cultures. The little fragment of explanted tissue was able to emit new cells when cut out of the old culture medium, washed in Ringer's solution and placed in a new fresh plasma. Carrel's thought was that senescence was an unnecessary

phenomenon which could be suppressed by freeing the cells from the catabolic substances in the exhausted culture medium and transfer them to a fresh medium.

Later Carrel⁵⁸⁾ found that certain tissue extracts accelerated the growth markedly and that especially the addition of embryonic tissue juice of the same species as the tissue cells to the plasma medium had an enormously growth-promoting effect. In this way it was possible to keep the tissues living *in vitro* indefinitely. The importance of having pure strains for experiments has already been mentioned.

In contrast to this technique, other investigators have been employing totally different techniques which are simpler in a way but give a field of investigation which seems to be far more limited. Lewis and Lewis who have developed this technique are not interested in having strains of tissue, but make their observations on one-generation experiments. This technique is limited to morphological studies of the tissue cells and is an excellent method for histological and embryological investigation. For this purpose much simpler culture media can be applied. Experiments of this kind will not give us much physiological information; because it literally is not a culture, but a mere survival of tissues under much too complex conditions. If one wishes to study the influences of certain substances, more or less harmless in character, it often takes time before they affect the tissues, just as well as the fresh, in saline solution explanted, tissues do not immediately die, on account of the juices which are brought to the culture medium by the tissue itself. There is a certain period of latency before the tissue becomes adapted to new environments.

The technique used for the cultivation of tissue cells has in the meantime, been simplified.

It is known that during a certain stage of the early investigations in tissue cultivation it was found that a slight hypotonic plasma-medium gave the best results. This does not

seem to be the case. Ebeling¹⁶⁰ has investigated the influence of different osmotic pressures on the growth of fibroblasts and found that decrease and increase in osmotic pressure resulted in a lower growth rate. Apparently the width of the area of new cells grown in a hypotonic culture medium was larger, but thinner, so that the actual amount of new tissue was less.

For quite some time it was stated by Carrel⁴⁹ that when cultures were kept under conditions in which two phases alternated, a phase of active life in a plasmatic medium in the incubator, and a phase of latent life in Ringer's solution in the refrigerator, they could easily be kept alive.

The thought was this, that during the latent period of life all the catabolic substances could be removed and then be rejuvenated for the active period of life in the culture medium. But it was observed shortly after that a much more uniform growth could be obtained by a simple and brief washing in Ringer's solution and afterwards being transferred to a fresh medium.

The technical part of the culture work, as it according to Carrel is carried out today, shall be described.

PREPARATION OF THE CULTURE MEDIA.

1. Preparation of the Plasma. The animal of which the plasma is to be taken is usually starved for a day or so previous to the blood being taken. For the cultivation of chicken tissues, the plasma is taken from adult chickens. The best plasma is obtained from young, healthy adult chickens not over two years old. If the chickens are not starved twenty-four hours before bleeding, the plasma obtained is turbid and rich in fat globules. Such plasma gives an unclear clot in which the cells do not develop as well as in the clear plasma. Under ether narcosis the blood vessel, artery or vein, usually one of the carotids, is dissected

free from the surrounding tissue. The wall of the blood vessel is cleaned with dry gauze and covered with olive oil. If an artery is used the part distal to the place selected for the incision is closed with a ligature and the part central to the place of incision is closed with a *serresfinēs*. The vessel wall is then opened by a slight cut with a pair of small scissors and a cannula, which previously has been sterilized in olive oil, is inserted into the lumen of the vessel. A ligature is then made round the blood vessel to fix the cannula tightly. The *serresfinēs* on the central part of the vessel is taken away and the blood now flows out of the cannula. The first few c.c. of blood are not collected but then the blood is allowed to flow down into paraffin-coated tubes, previously cooled at 0° C. The tubes are corked and centrifuged for five minutes. The supernatant plasma is removed with pipettes coated with paraffin. The plasma is stored in paraffin-coated tubes in an ice-box. In this way the plasma from chickens can be kept fluid for several weeks.

2. Preparation of the Embryonic Tissue Juice. Fresh fertile eggs are incubated in the usual way at a temperature of about 39° C. The air in the incubator is allowed a certain humidity. Every day the eggs are moved to prevent the embryo keeping the same position all the time. When the eggs have been incubated about 4-7 days, they are candled to see if they are good.

The embryo used for making the extract are usually from 7 to 10 days old. The eggs are opened by cracking the shell on the upper quarter of the shell at the broad end. The shell is taken away as a little cap. This part corresponds to the air chamber of the egg. The snowy white membrane just under the shell is opened with a small sterile forceps and the amniotic sac appears with the rich vascular system shining through. With a small pair of scissors this sac is opened and one can now, with an iris forceps, grasp the little embryo around its neck and remove it

without touching the shell. In this way sufficient embryos are collected on a sterile watchglass. The embryos are washed with a little Ringer's solution which is drawn off again with a pipette. Then the embryos are cut up with a pair of small curved scissors, until a fine pulp is obtained and then this is placed in small narrow (5 m.m. in diameter) tubes by means of a pipette and centrifuged for ten minutes. The supernatant fluid in these tubes is then ready for use. The fluid is a little opaque and rather mucous. I have observed that the embryonic tissue juice obtained in this way has a much more activating power than if the embryos were ground with sand or kieselgur in a mortar.

It is always advisable to draw off the tissue juice from the pulp and keep it separately in the refrigerator. It keeps better and the hydrogen ion concentration keeps more constant for a longer period of time. The tissue juice loses its activating power and becomes rather acid if the tissue pulp is allowed to remain with the juice. Usually the tissue juice is prepared fresh every time it is to be used.

MAKING THE CULTURES.

The apparatus necessary for the complete culture work is the following:

- The plasma in paraffin-coated tubes
- The embryonic tissue juice
- Ringer's solution
- Tyrode's solution
- Rubber nipples for the pipettes (autoclaved in water)
- Pasteur pipettes
- Centrifuge tubes
- Big glass dishes, containing the hollow slides
- Coverglasses
- Cataract knives
- Steel needle on a shaft
- Coverglass forceps
- Iris forceps

Small straight and curved scissors
 Vaseline
 Glass rods
 Gauze, filter paper
 Black cloth for covering the table.

Formula for Tyrode's solution:

NaCl	8.0	grams.
KCl	0.20	..
CaCl ₂	0.20	..
MgCl ₂	0.20	..
NaH ₂ PO ₄	0.05	..
NaHCO ₃	1.0	..
Glucose	1.0	..
H ₂ O	1000.0	..

Formula for Ringer's solution.

NaCl	9.0	grams.
KCl	0.42	..
CaCl ₂	0.25	..
H ₂ O	1000.0	..

Method of cleaning slides, coverglasses and pipettes.

Boile for 10 minutes in water with ordinary unperfumed handsoap. Allow luke-warm water to run until all soap has disappeared. The glasswares are placed in 96 % alcohol and hereafter polished with a clean towel.

It is hardly necessary to say that all of the utensils have to be sterilized in the proper way.

The room for the operations must be clean and not too cold.

The pipettes are supplied with rubber nipples and filled with the respective solutions, plasma, tissue juice and Ringer's solution and kept on the table under cover of the sterile centrifuge tubes, which are placed on the table.

The coverglasses are laid on the table and a drop of plasma delivered from the pipette which has to be kept strictly vertical in order to deliver even-sized drops. The pipettes are calibrated so that they all deliver drops of practically the same size. The plasma is spread out with the cataract knife so that it covers an area of about 10–15 m.m. in diameter. With the point of the knife the little fragment of tissue to be cultivated is placed in the plasma and a drop of the embryonic tissue juice is added and the mixture stirred with the knife. Small amounts of vaseline are placed on the hollow slides and they are inverted and placed over the coverglass lying on the table. This has to be done rather quickly to avoid too much evaporation from the culture media. After a minute or so the coagulation of the culture medium has taken place and the slides with the cover glass can be taken up and sealed with paraffin. Immediately after the preparation they are placed in the incubator 39° C. Here they are allowed to grow undisturbed for 18 hours, after which time they are transferred to a fresh medium. The coverglass is lifted with the heated point of a blunt cataract knife. It is then placed upon a piece of black glass or similar material of about one inch thick. It is necessary for cutting the culture to be able to lower the wrist sufficiently to place the cutting edge of the knife parallel with the surface of the culture. The tiny tissue-fragment and its crown of new growth can be seen better on the black background. The fragment is extirpated by four clean cuts made with the blade of a sharp cataract knife within the area of new growth and divided into two or three pieces of as equal a size as possible. The pieces are transferred with the point of the knife to an oval slide containing Ringer's solution. They are allowed to remain in the bath for about 15 seconds. In the meantime the new medium is prepared on the coverglasses in the way just described and the fragments placed in it. When many cultures have to be changed, it is safest to take a new knife

for every 4 cuttings or so to avoid bringing possible infectious material from one culture to another.

The fragments of tissue are transferred by means of the knife point to the medium. They must be embedded thoroughly in it without folding or curling. This step must be carried out rapidly to guard against embedding after coagulation has set in. In between the manipulation the preparations are kept under a large Petri dish in order to avoid bactericidal contamination from the air.

This technique briefly described here can be applied to the cultivation of all kinds of tissues in general. Slight modification may be necessary though in general the principles are very much the same. It will be described in detail in another chapter.

In the meantime, several investigators have tried to simplify the culture-technique by operating with simpler and less complicated media. As mentioned often before, Lewis and Lewis³³⁷⁾³⁵⁰⁾ used different salt solutions with the addition of chicken broth, agar, and so on; Swezy⁴⁹⁰⁾ used a medium composed of egg albumin and muscle extract. The growth obtained in these various media was not equal to that in plasma or lymph. Ebeling and Carrel found that the fibrin has no other value for the cells than to serve as a scaffold for new cells. Ebeling¹⁶²⁾ worked out a technique by which the medium was prepared from fibrinogen, serum and tissue juice and quite recently by using only fibrinogen, tissue juice and Tyrode's solution, or fibrinogen, egg albumin and tissue juice. In this way it was possible to get rid of the growth inhibiting factor in the serum. In the beginning there was some trouble in using fibrinogen, tissue juice and egg albumin, for liquefaction of the culture-medium occurred after a short period of incubation. But this was overcome by adding a trace of unsaturated fatty acids to the medium. Sodium linoleate was used and no liquefaction occurred. The investigations mentioned have just begun and can only be looked upon as being of great value and importance.

The preparation of the fibrinogen is carried out *ad modum* Mellanby ¹⁴¹ or by precipitation with purified CO₂. The fibrinogen suspended in a small amount of distilled water had the appearance of rich milk. This suspension could easily be redissolved in serum or Tyrode's solution and by the addition of embryonic tissue juice to the fibrinogen serum or fibrinogen-Tyrode solution gave a clear firm clot. The growth in a medium composed of 12.5 % fibrinogen suspension, 37.5 % serum and 50 % embryonic tissue juice was a little less extensive than in the medium composed of plasma and tissue juice. The advantages of using fibrinogen are numerous and will be still more valuable when the serum itself can be eliminated.

THE RATE OF GROWTH AND ITS ESTIMATION.]

It has often been observed that two fragments of the same tissue placed in identical media do not grow at the same rate and that these differences in rate of growth can be explained as technical errors. It is therefore highly important to know a little about these technical errors in order to be able to judge an experimental result. Ebeling ¹⁶³ has investigated this particular point and found that the experimental error in regard to such complicated experiments could be reduced to less than 10 %. Special care has to be taken in the selection of the cultures for experiment, preparation of the tissue and the preparation of the media according to Ebeling.

SELECTION OF THE TISSUE.

The cultures to be selected for experiments have to be in a healthy condition, i. e., the central portion must not be too thick and dense; there will often be a necrotic part. The necrotic portions can easily be detected by their complete opacity to transmitted light and they appear as white spots when seen by reflected light. The tissue for experiments is best when it has an even, good density of

healthy looking cells. Therefore, only strains which have been cultivated for a longer period of time in vitro (3 or 4 weeks) are good for experiments. Fresh tissue cannot be controlled, the size (area and thickness) cannot be measured easily and besides this it contains usually all kinds of tissue elements which complicate the experiments. On the contrary, tissue cultures with a big area of new growth, but scattered around in a loose meshwork with rather big spaces in between the cells, must be discarded for experiments.

PREPARATION OF THE TISSUE.

For experiments it is necessary always to have reliable controls. Therefore, the selected culture has to be divided into two or four parts and one of them serve as control for the others. It is necessary to make the control and experimental culture out of the same fragment: there may be differences in activity and structural conditions between two entirely different cultures. The tissue is extirpated from the coagulum by four clean cuts with a sharp cataract knife. *Any part of the coagulum which has not been invaded by the cells must not be left around the tissue.* If the incisions are not very clean, the coagulated plasma may become twisted and folded and when placed in the new medium, may prevent it from growing regularly. Hereafter the extirpated fragment is divided in as equal pieces as possible, washed in Ringer's solution and placed in the new media. If the difference in size of the new fragments is too great, this alone will cause a difference in rate of growth. It was observed by Burrows that the new cells travel further away from a big piece than from a small piece, probably due to the accumulation of more waste products in the larger piece than in the smaller.

PREPARATION OF THE MEDIA.

It is hardly necessary to mention that all chemicals used for preparing Ringer's solution have to be of the purest. The glass wares used for preparing the media must be such

that they do not increase the alkalinity of the fluids. The distilled water is redistilled in Pyrex or Jena glassware. The pipettes are made of alkali free glass. Mica is used instead of coverglass. It is important now and then to test the hydrogen ion concentration of the water, Ringer's solution, of the tissue juices, and so on.

PREPARATION OF THE CULTURES.

It is important to use calibrated pipettes for measuring the drops of plasma and tissue juice. It is absolutely necessary, in order to obtain comparable results, to use culture media of the same volume and same composition and allow the medium to occupy the same area on the coverglass. The growth varies much when the clot is of different thickness, firstly owing to the oxygen tension, and also due to the mechanical structure of the fibrin meshwork. To obtain clots with the same area, one can place the coverglass on a piece of paper on which a circle has been traced, or trace a circle on the mica if mica is used. After the plasma is dropped on the glass, it is spread out with the point of a knife in such a way that it covers the circle made on the paper. When the volumes of the media are the same and when the area which the fluid is allowed to take is constant, the thickness of the clot will be the same and the oxygen tension will be the same for the tissues in all cultures. The capacity of the hollow slides must also be identical. If one is larger the evaporation is greater and changes in concentration of the media occur. If one has to make experiments as to what effect, for instance, the dilution of the tissue juice has on the rate of growth, care must be taken to have all conditions the same as the one which is to be examined. If the influence of various substances on the rate of growth has to be tried and these added to the experimental culture it is necessary to compensate the dilution of the experimental culture by diluting also the culture medium of the control with *Ringer* or *Tyrode* solution in proportion.

MEASUREMENT.

According to Ebeling¹⁶³ the measurements can only be undertaken indirectly as we have no technique developed at the present time for directly measuring the actual increase in mass of tissue. In spite of the inaccuracy of the method, many new facts have been discovered. When proper care is taken, the thickness of the new tissue does not vary and can, therefore, be disregarded. The measurements of the areas of new tissues can be made in two different ways, either by measuring the width of the ring of new cells surrounding the original fragment, or by measuring the area of new growth. The last method gives more precise results. About an hour after the cultures have been prepared they are examined under the microscope to see whether they are still in good shape, whether they are cut neatly, whether there is any folding of the edges and whether the coagulation of the new medium has taken place and so on. Then the cultures are placed in a vertical projecting apparatus Edinger and the outline of the fragment is traced on a sheet of paper. This operation requires not more than 20 seconds. It is important to avoid overheating the culture while being projected. After 48 hours incubation a second drawing is made. Of course one has, in many cases, here to judge a little where to make the outline; sometimes the periphery may be a little irregular, and this, naturally, does not mean to trace single cells which may have migrated a little further than the rest of the big mass. If the outlines of the new growth are very irregular, some mistake has been made and the culture should be rejected. Afterwards the area of the original fragment and the total area after 48 hours are measured with the planimeter and expressed in square centimeters. The total area minus the area of the original fragment is the absolute increase of the fragment. This area, divided by the area of the primitive fragment, is the relative increase of the fragment. The relative increase of the experimental fragment divided by

the relative increase of the control fragment expresses the quotient, growth index.

Ebeling¹⁶⁾ showed, as mentioned, that if two fragments derived from one culture are cultivated under such uniform conditions as described above, the differences obtained are less than 10 %. It requires, of course, a fairly long period of practice and patience to be able to trust one's technique.

As it has been pointed out by Ebeling¹⁶⁾, the value of the growth of a piece of tissue is compared only with the value of the growth of another piece of the same tissue. Therefore, the absolute value of the growth is not of great importance and factors which do not modify the relative value of the growth can be disregarded. The factors referred to are the different qualities of the plasma, tissue juices, variations in the temperature in the incubator, and so on.

Several investigators have attempted to develop a technique by which it should be possible to maintain tissues in a condition of uninterrupted growth for long periods of time, (Romeis¹⁵³⁾, Burrows²⁸⁾, Fischer^{*)}. The technical difficulties were rather great, therefore, the principle of a method of cultivating tissue cells in vitro has been given up for a while. Quite recently Carrel⁶⁶⁾ has taken up the problem and worked out a method, although far from perfect, but indeed very important and useful.

The problem consists of supplying the tissue cells with the necessary food and at the same time removing the decomposition products from the culture without disturbing the tissue and without bacterial contamination.

Burrows²⁸⁾ and Romeis¹⁵³⁾ have constructed complicated apparatus which allow a continuous current of solutions containing nourishing substances to overflow the tissue. I* have applied thin discs of elderberry pith as support for the growing tissue while the pith was

*) Fischer A., unpublished experiments.

floating on embryonic tissue juice. Once a week or so, the fluid, containing the catabolic substances was aspirated by means of a pipette and replaced with a new fresh fluid containing the food materials. None of the methods have had any practical applications so far.

Carrel ⁶⁶⁾ solved the problem by constructing containers and instruments for the special manipulation of the cultures. The principle is, that the culture medium is composed of two parts, solid and fluid; the solid medium is continually bathed by the fluid medium, which is changed as often it is necessary.

The containers are flat, round flasks with a 3 cm. long and 1 cm. wide oblique necks through which the media may be introduced and removed. The neck is closed with absorbent cotton and a rubber cap. Five types of flasks have been constructed so far. One type has an opening (3 cm. in diameter) at the top, and is used for the cultivation of tissues which cannot be handled conveniently through the neck of the flask. The opening is closed by a disc of glass or mica. Another type has two necks opposite each other to use when bimanual operation is necessary. Another type has a bottom opening closed by a thin mica plate and a top opening. This type is used when the culture has to be examined by the high power microscope during the cultivation. The culture can be removed by the mica plate at the bottom, fixed in formaldehyde, stained and studied under high magnification.

The culture medium is composed of two parts, solid and fluid. The solid medium is obtained by the plasma clot or fibrinogen, and the fluid medium consists of thin solutions containing the food material.

The plasma is introduced first (0.5 cc. generally) and allowed to moisten the whole bottom. Then 1.5 cc. of Tyrode solution containing 5 per cent tissue juice, is introduced and mixed with the plasma. Before the coagulation takes place, the fragments of tissues are introduced

by means of a long platinum spatula. After the coagulation has taken place, the fluid medium is poured on the surface of the clot — and the neck is flamed and closed by the cotton and a rubber cap.

The fluid medium is changed every 2 to 4 days according to the nature of the culture. The fluid is aspirated by means of a pipette or an aspirator and the new fluid introduced. It is claimed by Carrel⁶⁶ that it is possible to handle about 60 flasks in one hour.

The measurement of the rate of growth is undertaken exactly in the same manner as described for the hanging drop cultures.

By this method it has been observed that when the area of growth of connective tissue or epithelium is plotted in the ordinate and the time in abscissae the curve expressing the growth is a parabola. This is only the case, when the fluid medium contains nutrient substances. If the fluid medium was composed only of Tyrode solution or serum, in other words, a non-nutrient medium, the growth represents the residual activity of the tissue and the curve is generally an S-shaped curve.

The usual accidents by this method may be traced back to bacterial contamination. Special care has to be taken, when the same containers have to be handled several times during an experiment — but experience has taught that bacterial contamination can be avoided when the proper care is taken.

A culture of fibroblasts, epithelium or lymphocytes can be kept in a condition of uninterrupted life for about 3 weeks by this new method.

Besides the methods for cultivation of tissue cells in vitro already described, there are still several methods of explantations which are mere survivals of tissue cells in vitro and cannot be called tissue cultivation and are used by many investigators; it is not important and necessary

to describe all variations of these methods, but only to mention the prototype for the technique namely that developed by the Lewis's. For the cultivation of tissues from cold blooded animals, the technique of Braus²⁰⁾, Uhlenhuth¹⁹⁷⁾, Erdmann¹⁷⁸⁾ is beautifully described in their respective papers.

Lewis and Lewis use as mentioned before, fluid media for their explantation experiments. The so-called Locke-Lewis salt solution for cold blooded animals is the following:

NaCl	0,7	grams
KCl	0,042	„
NaCl ₂	0,025	..
NaHCO ₃	0,02	„
Glucose	0,25	„
Aqua dest.	90,0	cc.

Cultures of tissues from warm blooded animals were usually cultivated by Lewis and Lewis in Lockes solution:

NaCl	0,9	grams
CaCl ₂	0,024	„
KCl	0,042	„
NaHCO ₃	0,02	„
Glucose	0,5	„
H ₂ O	100	cc.

Of this solution is taken 80 cc. to 20 cc. chicken broth. The hydrogen ion concentration of the solution were usually pH 6,8 to pH 7,2.

The technique of Lewis and Lewis is simply this. On a cover glass is placed a small drop of the Locke-Lewis solution of the above composition and in the drop is placed the small bit of tissue. The cover glass is quickly converted over the hollow-ground slide and is closed with paraffin. In order to get a good contact between the ex-

planted tissue and the cover glass, which serves as support for the migration of the cells. I usually place the slides in the incubator upside down, in other words the culture chamber rests on the cover glass in the incubator. The tissues will then be at the bottom, i. e. towards the cover glass and a good contact between the tissue and the support is established. If this precaution is not taken, the tissue fragment will in most cases be found floating in the bulk of the drop and the cells are not able to migrate.

The technique of micromanipulation which has been applied to the tissue cultivation should be mentioned here.

Various substances can be inoculated into the culture medium during the observation of the living tissue cells in the incubator. Brauns²⁰⁾ has used this technique a good deal and by means of the finest Speemann's needles he was able to cut growing cell elements.

By means of the Barber's^{10, 11} micro-dissection technique or still better with that by Chambers¹¹⁹ modified Barber technique, it is even possible to inoculate various substances into the cytoplasm of a living tissue cell. It is also possible to introduce living bacilli into the protoplasm of the tissue cells. By the Chambers^{120, 121)} method parts of the body of the living tissue cell can be excised; the nucleus may be cut in two or single chromosomes may be removed.

For obtaining good photographs of tissue cultures, the Lewis and Lewis cultures give excellent results because the cells are in one single layer, namely on the cover glass, and because everything but the tissue cells can be removed from the cover glass.

It is more difficult to obtain good microgrammes from tissues cultivated in plasma media. Here we often get the plasma coagulum tinted at the same time as the cells

and consequently the contrast between cells and medium is not always the best possible. Another disadvantage in making photos of tissue cells in plasma media is, that the cells are seldom in the same plane and therefore difficult to focus on.

It is necessary to have a good deal of experience before good microgrammes of tissue cultures can be obtained. Photomicrogrammes of living and unstained tissue cells in cultures are rather difficult to take. The illustrator at the *Rockefeller Institute* L. Schmidt has contributed much to this particular technique. By special illumination from above the specimen, beautiful photos of living tissue cells have been taken by Schmidt.

The technique of micro-cinematography has been applied rather much to tissue cells in vitro. The master in that field being Comandon^{111, 112}. Also Braus²⁰ has been doing that kind of work with great success.

PREPARATION OF TISSUE CULTURES FOR MICROSCOPICAL PURPOSES.

The living, unstained culture is a good subject for microscopic examination, although not everything can be seen, nevertheless high magnification can easily be applied to the tissue cells within the culture chamber. It is advisable to have a microscope with a heating device so that cultures and single cells within the cultures can be followed for a long period of time without allowing the culture to cool off.

We will now describe some of the simplest and most suitable fixing and staining methods for the histological investigation. All the many special staining methods can, of course, be applied with some modification of the technique, but they will be disregarded here. Several beautiful methods of preparing the tissue cultures are described in the "Praktikum" by R. h. Erdmann. In the cultures we

always deal with the coagulum and therefore modifications had to be made in the ordinary staining processes in order to avoid any trouble from the clot. In many cases, therefore, the clot has to be eliminated. This is sometimes very difficult; at the same time, as the clot is dissolved, the cells may disappear too, when the specimens are treated further. Some investigators, such as Lewis and Lewis, have a beautiful technique for staining, as they do not use plasma clot for cultivation. As the culture work is taken up here from an entirely different point of view, a physiological, the clot is always present and special methods have then to be developed in order to follow the morphological changes in the cultures during the experimentation.

The vital staining is made use of a good deal in the culture work. The methods for doing it are simple and need only a few words derived from experience. The stains used are the usual: neutral red, methylene blue, trypan blue, Janus green, and Janus black and so on. The stain can be added to the plasma before coagulation has set in, or before the growth had begun, or it can be applied by opening the culture, after it has already grown for some time, and adding a drop of the staining solution on top of the plasma clot and resealing the culture immediately after. The concentration of the dyes used is such that the stain is present in the culture medium in about 1–20,000 or 40,000. In this concentration most of the dyes are harmless to the tissue cells. They can often be cultivated for several passages in a medium containing the dye. For photographic purposes, the vital staining method is very good for obtaining characteristic pictures of living cells.

The fixation of a tissue culture is done best in Ringer's solution containing 2 % formalin. One hour in this solution is sufficient for the fixation. The alcohol or sublimate fixation cannot be applied because the plasma clot becomes very opaque and intransparent.

The scheme for the rest of the preparation is the following when hematoxylin, for instance, is used for staining:

- (1) After fixation, washing in running water 3 hours at least.
- (2) Distilled water one hour.
- (3) Hematoxylin (Mink's and Paul Meyer's modification) 50—60 drops in 50 c. c. of water 10 to 12 hours.
- (4) Washing in water half an hour.
- (5) Five minutes in 50 % alcohol.
- (6) Five minutes in 75 % alcohol.
- (7) Five minutes in 95 % alcohol.
- (8) Ten minutes in a mixture of 95 % acetone and 5 % xylol.
- (9) Ten minutes in a mixture of 70 % acetone and 30 % xylol.
- (10) Ten minutes in a mixture of 30 % acetone and 70 % xylol.
- (11) Fifteen minutes in xylol (I).
- (12) Fifteen minutes in xylol (II).
- (13) Embedding in *balsamum canadensis*.

Another method must be mentioned, which gives excellent results because of the fact that the clot is decolorized in the acetone-xylol mixture and allows of a beautiful differentiation between the stained cells and the colourless plasma-clot. Besides that, the following method does not give any precipitation in the culture, what the hematoxylin often gives. The dyes used here are methylene- or azur-blue.

1. Fix in 2 % Formalin-Ringer's solution one hour.
2. Running water 3 hours.
3. Five minutes in distilled water.
4. Flood the coverglass with Loeffler's alkaline methylene blue and hold it over a pilot light until vapor appears. Remove from flame and allow stain to remain on specimen for about fifteen minutes; then pour off the stain.
- (5) Wash with water from a Pasteur pipette.
- (6) Dehydrate in alcohol: 50 %, 75 %, 95 %, one minute each.
- (7) Acetone-xylol mixtures (5 %, 30 %, 70 % xylol) two minutes each.

8 Pure xylol 2 changes two minutes each.

If the clot is very thick it is necessary to dehydrate longer in xylol-acetone.

The methylene blue solution is prepared in the following manner: Saturated alcoholic solution of methylene blue 30 c. c.

KOH 1 10,000 100 c. c.

The methylene blue is allowed to ripen in the incubator for a month.

The azur-blue solution is prepared as follows:

Azur II 0.3 gram

Alcohol 95 % 10 c. c.

The two staining methods just described are very satisfactory for the standard work and especially the methylene blue method gives very good pictures for microphotographic work.

If one has to investigate the intimate structure of the cultivated tissue, sections can be made, and this is a very valuable method for studying the relations of the cells to the entire clot.

Sections of the cultures are made in the usual way. The cultures are fixed on the cover slip and they can then be removed carefully with a scalpel and passed through the different dehydrating solutions and finally be embedded in paraffin. Collodion can, of course, also be used and is often easier to use for such small delicate subjects as are the tissue cultures.

Cohnheim's idea of using gold impregnation method on the corneal cells, in order to get the characteristic outlines, suggested that this method perhaps could be applied in staining the cells in tissue cultures. I tried the gold method of Löwitt's in the following way. In a dark room the coverslip bearing the plasma clot and tissue culture was put into concentrated formic acid for 2—3 minutes and

thereafter directly in 1 % gold chloride solution for about 5 minutes; then washed quickly in distilled water and put back into a solution consisting of equal volumes of formic acid and water for another 24 hours, also in the dark; after this washing, dehydrated in the usual way and mounted in balsam. This method showed a great advantage in that, by treating with acid, all the culture medium disappeared and only the tissue was left adhering to the coverglass. The clot was brought into solution as acid albumins by the formic acid. This is a great advantage because the clot always hinders more or less in finer microscopic examinations and especially when ordinary anilin dyes were used, the clot itself absorbed the stain and the contrast between the tissue cells and clot was less pronounced. For photographic purposes this method proved most excellent. No precipitation occurred, the cells were clean, and contrasty as if they were washed clean from all substance save the cells themselves.

The cells appeared reddish, more or less deep coloured, granules deeply stained and vacuoles and nuclei pretty clear and unstained. The outlines of the cells were usually in the most perfect condition and the finest, tiniest processes and connections in between the cells could be distinguished very sharply.

By using Löwit's method as described above there were some disadvantages. In most cases the dissolution of the clot was so vigorous that often the cells disappeared at the same time; therefore I modified the method. The successful preparation depends very much upon the location of the tissue in the clot; if the tissue is located near the free surface or in the middle of the clot, it will easily be washed away, when the clot is dissolved, but if the tissue is located near the bottom of the clot, i. e., close to the glass, it sticks to it and remains. The preparation method was modified in the following way. Instead of using the concentrated formic acid as a fixative, it was used as a 50 % solution and the so-

lution for reducing the gold chloride was 1 volume formic acid to 9 volumes water. In this solution the culture only remained for 24 hours and was then ready for mounting. This modification gave just as good staining results and was much safer. In most cases the central portion of the culture is lost, but this is of little consequence because no details can be observed in that part of the culture; it appears always as an opaque black mass.

Lewis and Lewis often fix their special cultures in Zenker's solution without the acetic acid, sometimes they add a little osmic acid just before using it. The Zenker's solution is composed of: potassium bichromate 2 per cent, sodium sulphate 1 per cent, corrosive sublimate 5 per cent.

Quite usually Lewis's cultures are fixed with iodine vapor by placing a small flake of pure iodine in the bottom of the hollowground slide. The iodine vapor rapidly tints the cells yellowish brown.

V.

PURE STRAINS OF TISSUE CELLS.

Soon after a technique had been developed for cultivating tissue in a way somewhat similar to the technique for cultivating bacteria, it was desirable to develop a method by which it would be possible to obtain cultures of tissue cells belonging to a definite type.

Carrel⁵³⁾ found that when a fragment of tissue had undergone several passages, in vitro, the outgrowth of new cells could easily be distinguished and a large group of cells belonging morphologically to the same type of cells could be separated from the other cells by cutting out the part of the fibrin film containing the cells desired and transferring it to a new medium.

There has been much discussion about dedifferentiation of cells in vitro, i. e., that the various tissue cells, whatever they may be, mesenchyme, or ectoderm, were said to be transformed into an embryonic indifferent type of cells. It was therefore necessary to find methods by which it would be possible to work with absolutely pure cells from the very beginning, in order to see if such transformations occur. Much has been written about the identification and classification of the different cells cultivated in vitro¹⁴³. The problem is brought out in connection with an assumption by Champy¹²³⁾, that a dedifferentiation of the epithelial cells to the type of fibroblasts takes place in vitro. This does not seem to be correct and is rather to be regarded as an apparent phenomenon. As long ago as in 1898, Leo Loeb³⁷²⁾ came to the same conclusion as Champy by observing the changes which epithelial cells undergo when cultivated

in the blood coagulum *in vivo*. Later Loeb³⁷¹ also advanced the opinion, that these cells do not dedifferentiate.

Champy¹³⁰ cultivated kidney tissue and found, that its structure changed: indifferent tubules were formed, which later on lost their renal and finally their epithelial character.

Uhlenhuth¹⁹⁹ showed, that tapetum cells from the retina changed their typical epithelial character with modifications in the consistency of the culture medium.

Chlopin¹³⁷, in his recent paper was not able to support the assumption of Champy. I have already observed myself Fischer¹⁹² that epithelial cells cultivated on the free surface of the coagulum did not change morphologically after more than four months' cultivation *in vitro*. This has been entirely confirmed by Ebeling¹⁶⁵, as he had a strain of epithelial cells for more than 18 months. As soon as the epithelial cells were allowed to grow embedded in the plasma clot, the individual cells changed from polygonal to fusiform cells. In order to find out truly what happens to the different tissue cells when grown *in vitro*, it is absolutely necessary to be certain of the kind of cells we are operating with. At the present time no method is known by which it is possible to isolate single cells for obtaining pure strains, as it is known from the bacteriological technique. As described in another chapter, a technique was worked out to isolate cells; but single cells do not seem to develop and multiply unless they are in contact with several other individuals. This method can, therefore, not be applied.

There are two methods by which it is possible to obtain pure cultures of tissue cells — the one, by which the tissue cells for cultivation are obtained pure from the very beginning, i. e. an anatomical method, — the other, more generally applied, to be used only for certain cells, may be called a physiological elective method. If a piece of heart, for instance, is explanted in a certain medium, the muscle cells and a few other cell types die out rather rapidly and the fibroblasts will continue to grow

and multiply, and finally are obtained pure. The same method is used in getting the big mononuclear lymphocytes. When leucocytes are cultivated, all the different elements of cells will die out except the big mononuclear lymphocytes, which will persist. (Carrel and Ebeling⁹⁶). The method is somewhat similar to the different elective methods for obtaining pure cultures of certain bacteria, as for instance, the diphtheria bacilli by cultivation on serum, the typhoid bacilli by the bile method, and so on. The horse serum or the bile is not used because diphtheria bacilli grow better in horse serum, or typhoid bacilli grow better in bile, but because other bacteria grow very poorly on these substances. The same thing is probably the case with epithelial cells, polymorphonuclear leucocytes, and so on; they do not grow so well under the same conditions as the fibroblasts.

The other method for obtaining pure cells for cultivation, the anatomical, is simply done by dissecting the pure tissues out from the organism. The tissue cells for cultivation have to be taken from certain anatomical *loci electi*, where we know that these tissues are pure, i. e., only contain one type of cells. Rather few tissues are pure. The majority of them are complex, containing ameboid cells, vessels, glands with their various cell elements. Cartilage is a tissue which can be obtained pure that way. Epithelium is more difficult to obtain because it is often so closely adherent to the endothelial and connective tissue cells; nevertheless it is rendered possible to obtain pure epithelium, from certain *loci electi*.

FIBROBLASTS.

The connective tissue strain which has been cultivated for more than 12 years in the Rockefeller Institute in New York, (Fig. 8), and for 3 years in the Institute of General Pathology of Copenhagen were isolated from a complex tissue, namely a piece of embryonic heart. After a certain length of cultiva-

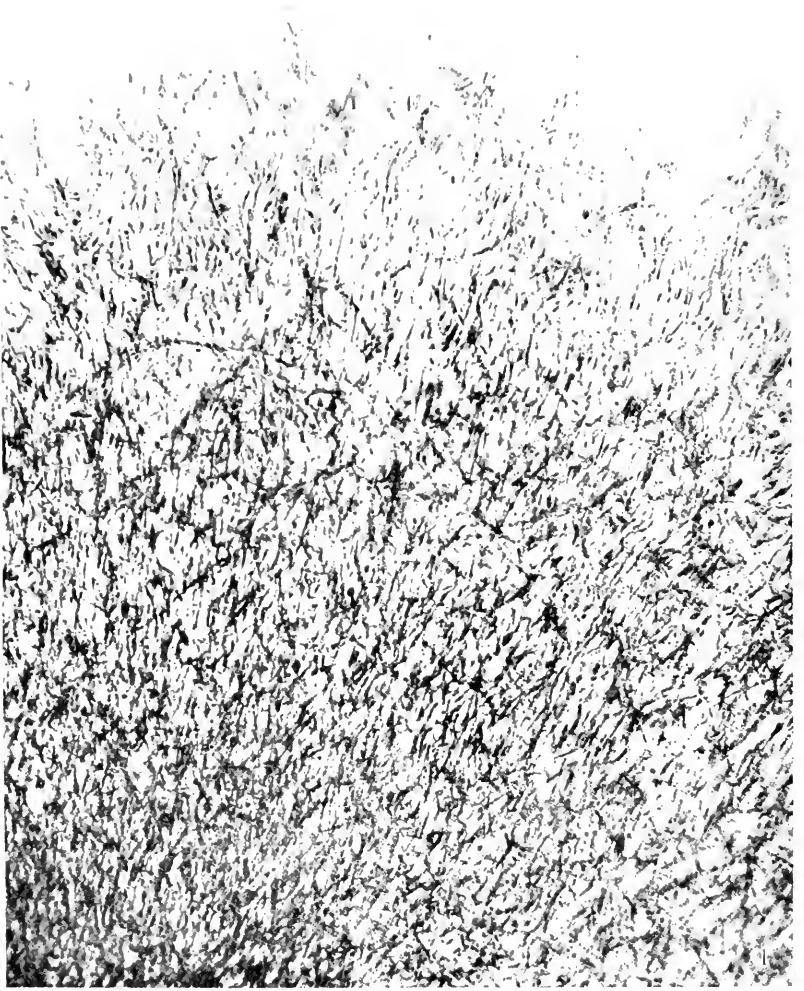


Fig. 8.

From Ebeling: A ten year old strain of fibroblasts.
Journ. Exp. Med. 1922, XXXV, 231.

tion the muscle cells, ameboid cells, and so on disappeared and the endothelial cells or fibroblasts remained. This method for obtaining pure cultures of fibroblasts, the physiological elective method, is good and safe because all the other cell types do not grow so rapidly or as well as the connective tissue cells which sooner or later overgrow any of the other types of cells. Cultivation of this tissue has now been enlarged upon so thoroughly that there is no need to go more into details about it. The strains of connective tissue cells have been used as a reagent for the detection of substances in the humors of the body, which have the power of increasing or decreasing the rate of growth and many biological problems have been studied with the fibroblasts as reagent and references are already made to the various papers on these subjects.

EPITHELIUM.

The growth of epithelium *in vitro* has been described very often.

Several attempts had been made to obtain strains of epithelium for permanent cultivation outside the body, as has been carried out with the connective tissue cells. From the very beginning of the experiments of cultivation of tissue cells *in vitro* — the cultivation of epithelium has tempted most investigators. It has been possible to cultivate epithelium *in vitro* for short periods of time, long enough to observe and describe the characteristic growth of epithelium.

Most investigators, who have worked with tissue cultures, have not been able to keep strains of tissue cells over long periods of time, from months to years. Tissue cultures have mostly been studied in the culture of first passage — and kept under observation in the same culture medium for a few days only or a couple of weeks. Should it therefore be possible to cultivate a strain of epithelium for a long period of time, and the cells still remaining characteristic for epithelium all that time, first then one may conclude,

that the cells are pure and that no dedifferentiation has taken place.

Epithelium has been cultivated from human beings, dogs, cats, chickens and from several cold blooded animals Ruth¹⁹¹, Oppel¹³¹, Holmes²⁶³, Uhlenhuth¹⁹⁷ and Carrel*. Carrel* has often tried to obtain permanent strains of epithelium as is done with connective tissue cells, but after cultivation for a certain length of time the epithelial cells disappeared and fibroblasts began to appear. This invasion of fibroblasts was due to a contamination of the epithelial cells with fibroblasts from the very beginning at the time of explantation. It may have only been an apparent appearance of fibroblasts, i. e., a morphological change of the polygonal epithelial cells into a fibroblast-like type of spindle cells may have taken place. Champy¹²³) interpreted the phenomenon as a dedifferentiation. Uhlenhuth¹⁹⁸ explained the dedifferentiation as the results of the mechanical conditions of the culture medium.

TECHNIQUE FOR THE PREPARATION OF THE TISSUE.

The epithelium for the cultivation is obtained from that part of the iris which spontaneously adheres to the brim of the lens when it is removed. The lens is profitably taken from the eye of the 10-12 days old chicken embryo. The best way of extracting the lens, is to make a slight incision in the posterior pole of the eyeball and with a small forceps, the entire corpus vitreum is drawn out. Attached to the front of the corpus vitreum the lens will be found. The lens is now separated from the corpus vitreum. In most cases a thin black brim of the iris will be found on the lens. It is necessary to work on a background of some white paper or so, otherwise the black iris brim will not be seen. The lens is cut in 2 or 4 pieces and placed

*) Carrel, A., unpublished experiments.

in the culture medium. If the lens is not extirpated in the way described here, it may happen, that fibroblasts from the iris will remain on the lens and the cultures will then not be pure epithelium.

TECHNIQUE FOR THE PREPARATION OF THE CULTURES.

It was observed, that the epithelium grew very irregularly at the outset. An extensive liquefaction of the plasma occurred during the growth and after a certain amount of the clot had liquefied, the growth became very extensive. This fact suggested, that if we immediately could bring the fragment of tissue under such conditions as had a liquefaction already taken place, the result would probably be an extensive and uniform growth. It was therefore essayed to place the little fragment of epithelium on the free surface of the coagulated plasma and cover it with a thin film of embryonic tissue juice. The result of this arrangement was very striking. — The culture medium was composed of, as usual, equal volumes of embryonic tissue juice and plasma from adult chickens, which are added together on a coverslip and mixed. When the coagulation commenced which can be ascertained with the point of the knife, the tissue fragment is placed on the free surface of the nascent clot. It is important to place the tissue at the proper moment: if it is placed before the coagulation has commenced, it will be embedded in the clot — and if it is placed too late, it will not adhere, but float in the covering droplet of tissue juice. After the fragment is properly placed, a small drop of embryonic tissue juice is spread out to a thin film on the clot to establish a moist surface for the proliferation. When transferring the culture, the fluid is removed by means of a piece of steril filter paper, and the culture cut out of the clot in the usual way.

RESULTS.

After a period of latency 3-8 days the epithelium commenced to grow. Uhlenhuth⁴⁹⁷ also found, that some time elapses before the epithelium begins to grow.

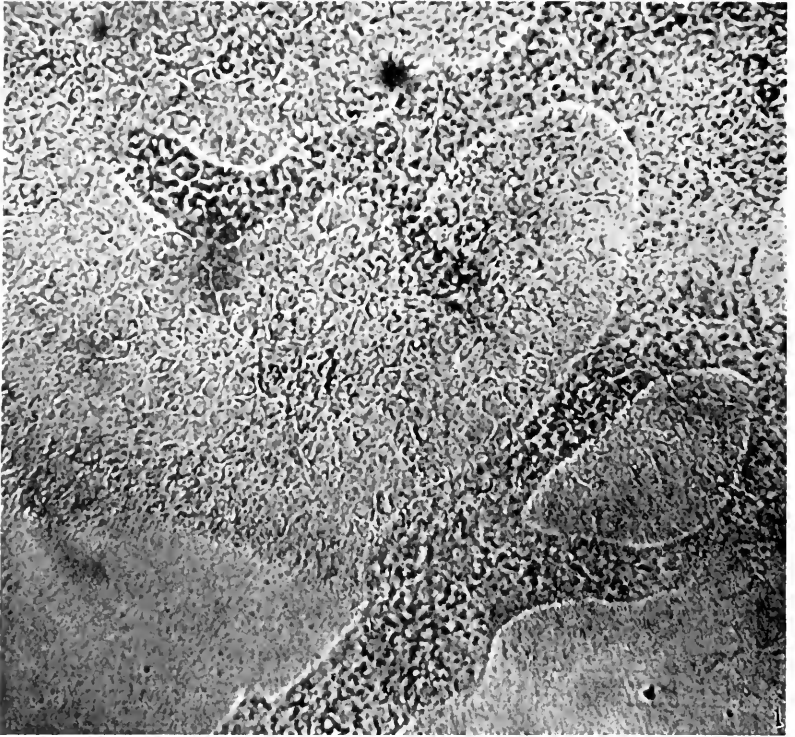


Fig. 9.

Photograph of a living culture of epithelium 6 weeks old. Shows the characteristic growth for epithelial cells. - about 135.

From The Journ. of Exp. Med. 1922, XXXV, 367.

To begin with, the epithelium grows with great irregularity, but with proper care, it soon commences to grow very abundantly. It will now be seen, that after a few hours incubation, the epithelium cells grow out from the fragment

and 48 hours afterwards an extensive uniform growth has taken place around the old piece. In cultivating the epithelial cells on the free surface of the clot, I obtained large

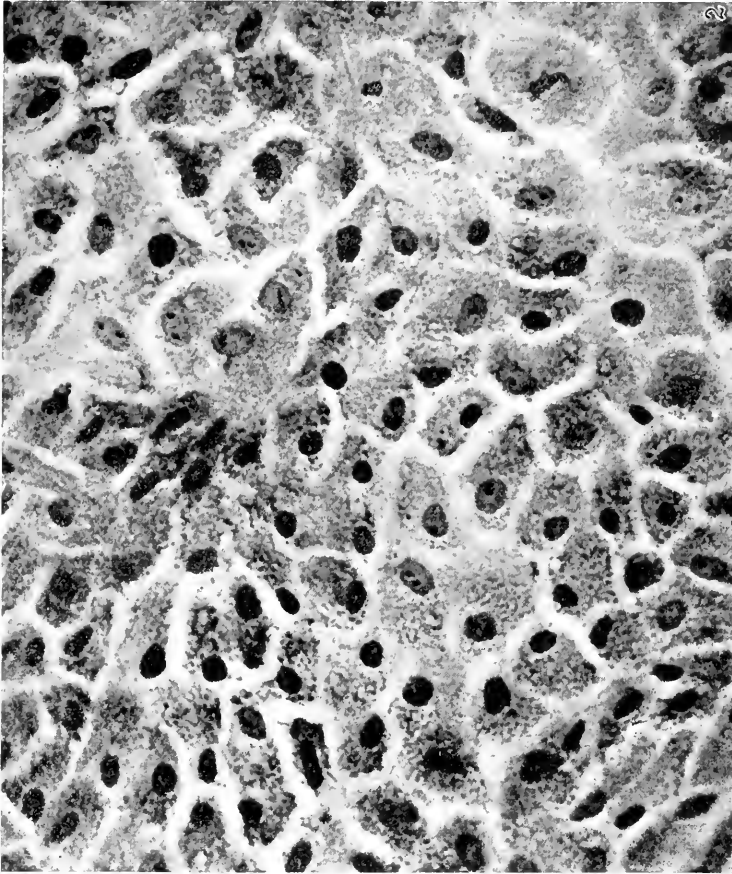


Fig. 10.

Stained culture of epithelial cells, 6 weeks old. Shows the epithelial pavement formation; mitosis may be seen. \times about 660.

From The Journ. of Exp. Med. 1922, XXXV, 367.

cultures which easily could be multiplied and what was still more important, I was able to keep the cells growing

under much more uniform conditions than would have been the case if the cells were allowed to grow embedded in the clot. The conditions on the moist surface are much the same as those under which the epithelial cells normally grow in the body.

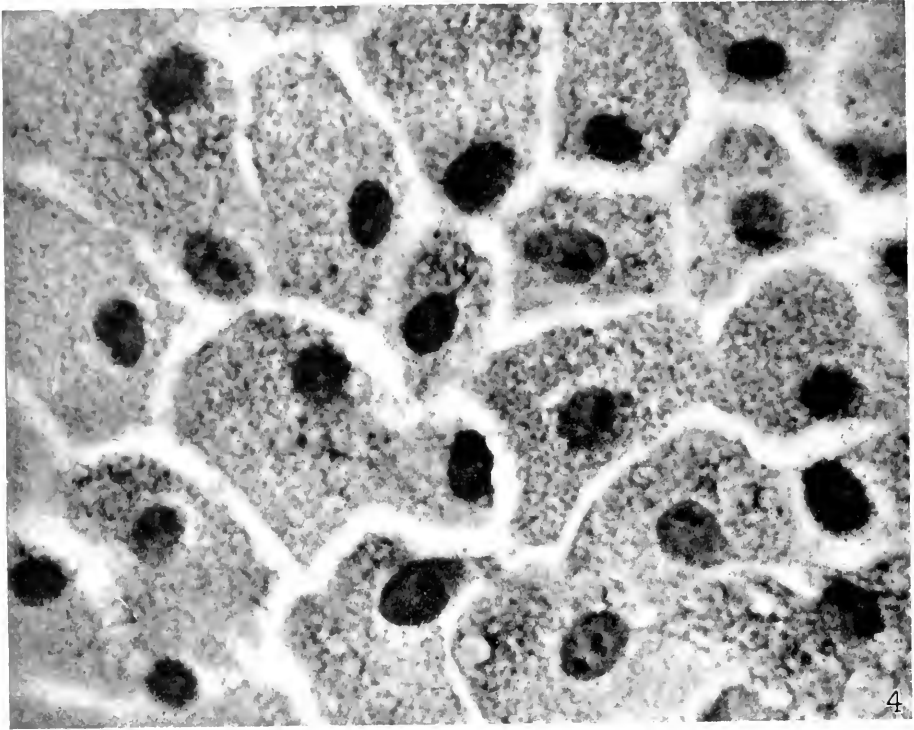


Fig. 11.

Stained culture of epithelial cells 2 months old. $\times 1,425$.
From *The Journ. of Exp. Med.* 1922, XXXV. 367.

The characteristics of surface cultures of epithelium are the following: The cells form big solid sheets or membranes, mostly in one layer only. Fig. 9. The cells are polygonal and form a pavement. Fig. 10. Between the individual

cells usually a little space can be seen, which is of pretty near the same size all over; in other words, the cells are apparently not in direct contact with each other, but leave open a small space of about one-fourth the size of the cell. By higher magnifications, it can be seen that the cells are connected by means of protoplasmic processes which are extremely delicate. Fig. 11. The cytoplasm is finely granulated sometimes filled with pigment. The nucleus usually contains one large nucleolus. Numerous mitotic figures can be beautifully seen, because of the unicellular layer. Fig. 12. In many of the surface-grown epithelial cultures the cells arrange themselves in rings and appear as a cross section of a gland; often this glandular arrangement can be seen formed around a giant cell or cells in mitotic division. Fig. 13. Some cultures, when stained, remind one very much of a strongly metaplastic epithelium, as it is known from certain malignant epithelial proliferations. All different shapes and sizes of cells can be observed in these cases; sometimes it can be directly seen how quickly the proliferation must have taken place. Such proliferation centres are not unusual and they frequently give an impression that a proliferation of an almost explosive character has taken place. Fig. 14. We have very frequently found two cells which had practically not quite separated by division before the two new cells showed new mitotic figures. Many stages of amitotic cell division can be seen in the cultures. When the culture of epithelium gets older, i. e., not transferred into a new medium after forty-eight hours, but allowed to remain in the same culture medium for a longer time, the cells can be seen with big vacuoles and often filled with pigment. At the same time giant cells appear. These cells may contain 10–15 nuclei and are many times larger than the normal cells. Around such a giant cell normal epithelial cells can be seen in fairly regular circular arrangement.

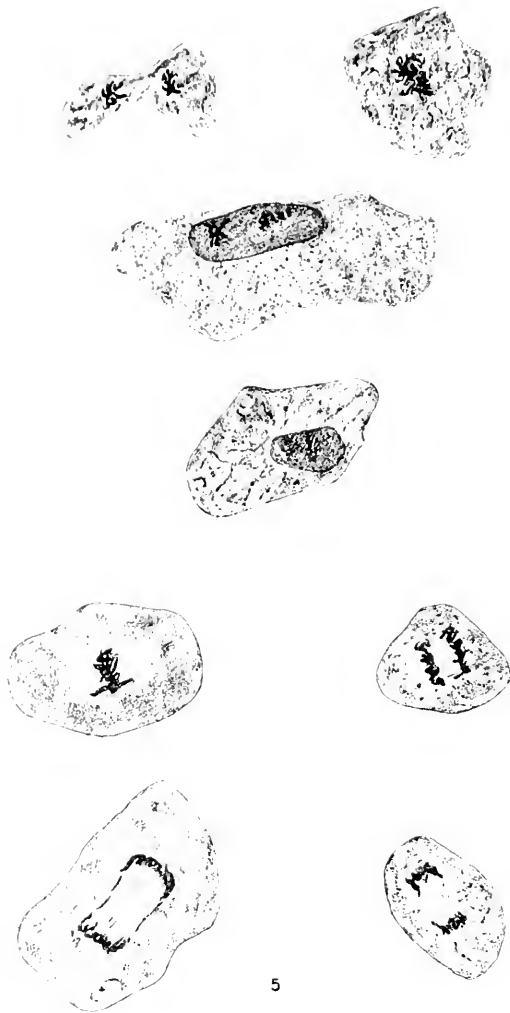


Fig. 12.

Different stages of mitosis of epithelial cells in cultures.

From The Journ. of Exp. med. 1922, XXXV, 367.

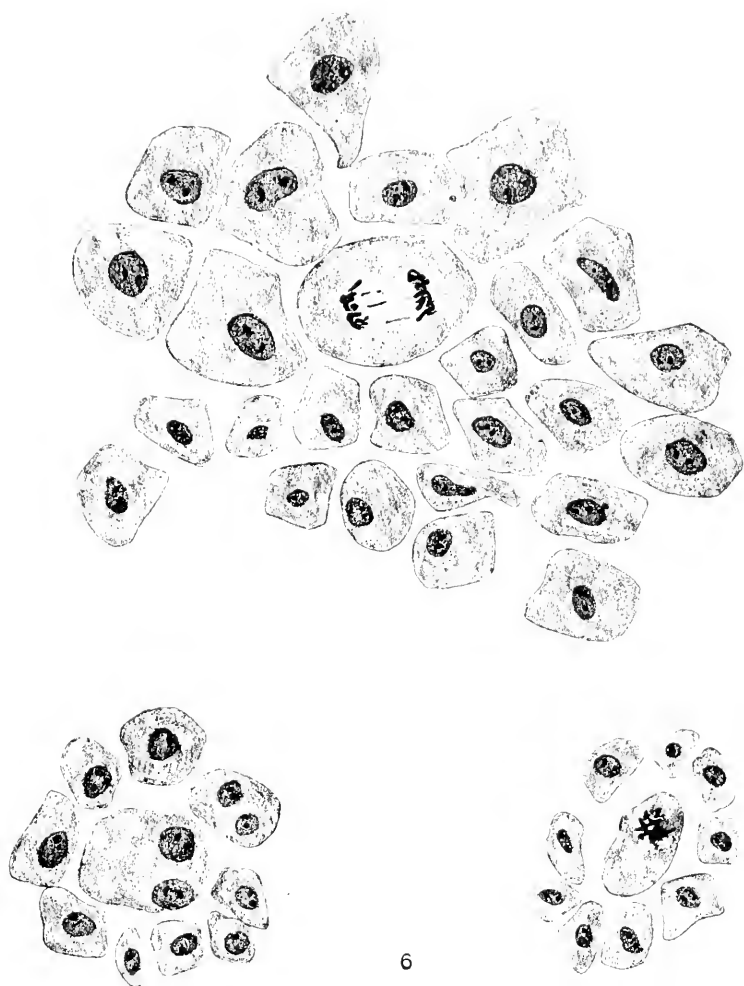


Fig. 13.

Typical cell arrangement in epithelial cultures. $\times 700$.

From The Journ. of Exp. Med. 1922, XXXV. 367.

In the periphery of the growth more elongated fusiform cells are often found. This is probably due to the fact that the cells have reached the border of the clot covered with tissue juice and therefore embed themselves in the clot and become spindle-shaped for that reason.

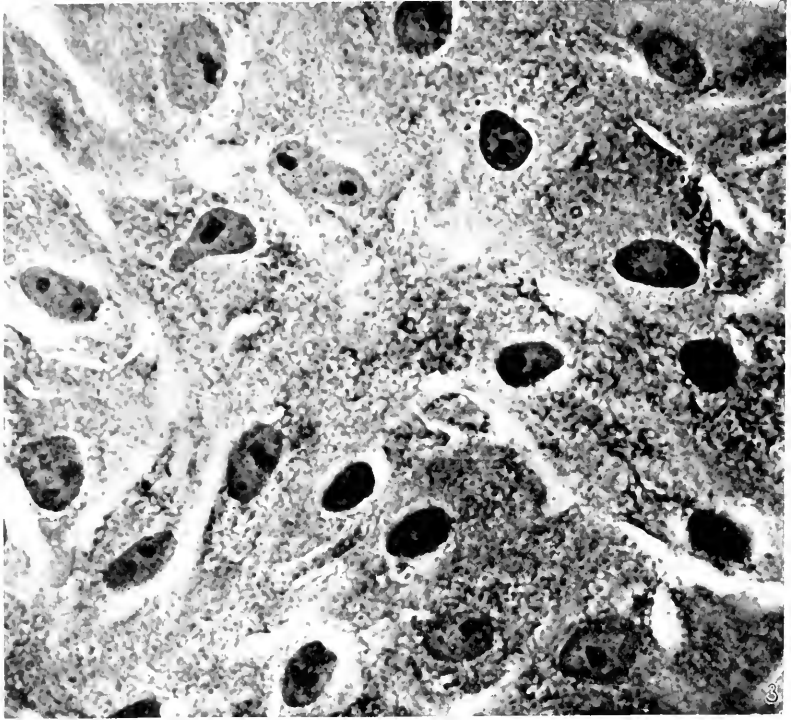


Fig. 14.
Stained culture of epithelial cells, 6 weeks old. 1,100.
From The Journ. of Exp. Med. 1922, XXXV, 367.

As mentioned before, Uhlenhuth¹⁹⁸ observed that changes in the consistency of the culture medium had a marked influence on the cell shape. In soft medium obtained by diluting with muscle extract the polygonal cells changed their form into a fusiform; and in a hard medium obtained

by adding chicken-plasma to the medium the epithelial cells grew out in big sheets of polygonal cells. This is an important fact and will probably explain a good many things in the morphology of cells. The mechanism of these changes in morphology, according to the structure of the solid phases in the culture medium has been subjected to several explanations. The medium which Uhlenhuth¹⁹³¹ calls soft is composed of a relatively small amount of solid phase, i. e., the fibrin fibrillae are very sparsely represented. In this medium the epithelial cells grow out on the sparse fibrin threads and consequently become spindle-shaped because of the relatively big spaces of liquid in between them. We know that the tissue cells must have a stroma to grow on, otherwise they become round and die. In the hard media we have a dense meshwork of fibrin threads, which acts more like one big solid phase; the cells are able to spread themselves out as epithelium naturally does. As the epithelial cells have been cultivated in vitro in between a liquid tissue juice and a solid fibrin phase, the cells appear as they do in the organism under natural conditions, namely, as a pavement in solid sheets, Fig. 10. When fibroblasts are cultivated on the surface of the plasma-clot, they immediately embed themselves in the medium. It seems as if the epithelial cells are inhibited by the mechanical resistance of the fibrin-fibrillae to a much greater extent than are the connective tissue cells. When the epithelium is cultivated embedded in the clot, the growth seems to depend very much upon the existing condition within the medium and the disposition of the fragment. If the condition of the coagulum prevents the uniform outgrowth of new cells from the mother fragment and when the condition of respiration of the fragment is not of the very best, then the cell-invasion is characterized by the formation of branching tubules of various forms, (Fig. 15) but essentially the arrangement of the growing cells is such as to form a more or less organized structure resembling hollow tubules. The

growth and migration of epithelial cells seem to be much more dependent upon the mechanical condition than those of fibroblasts. Leo Loeb³⁷⁴ has already pointed out years ago, that the epithelium follows the way of least resistance.

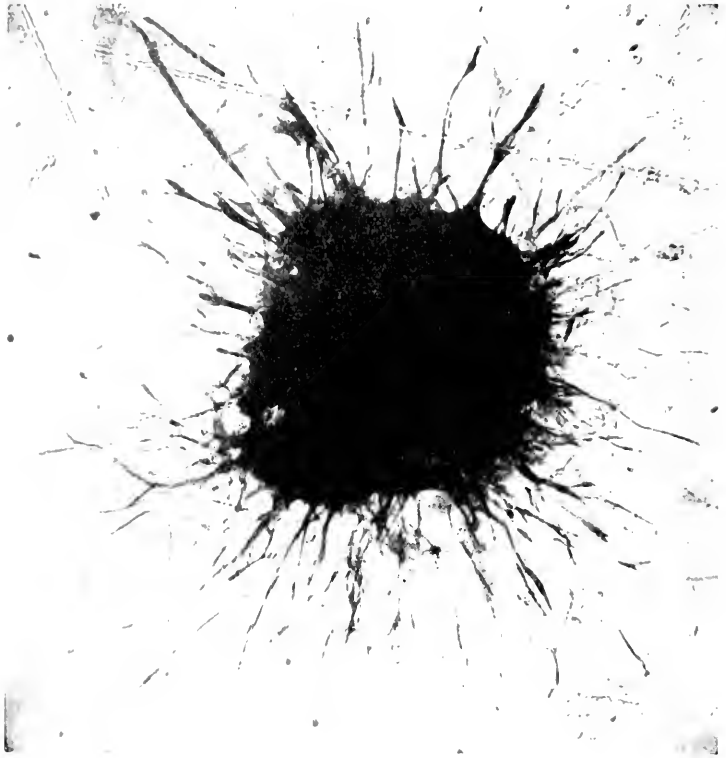


Fig. 15.

18th passage of a pure culture of epithelium 48 hours old, from the iris of the chicken embryos. The new growth is found as rudimentary tubules.
From The Journ. of Exp. Med. 1924, XXXIX. 585.

When growth occurs in membrane formation, it is rapid and extensive; when the tubular type results, the rate of growth is markedly slower and the actual increase in mass

is small, although the length of the tubular outgrowth may be extensive.

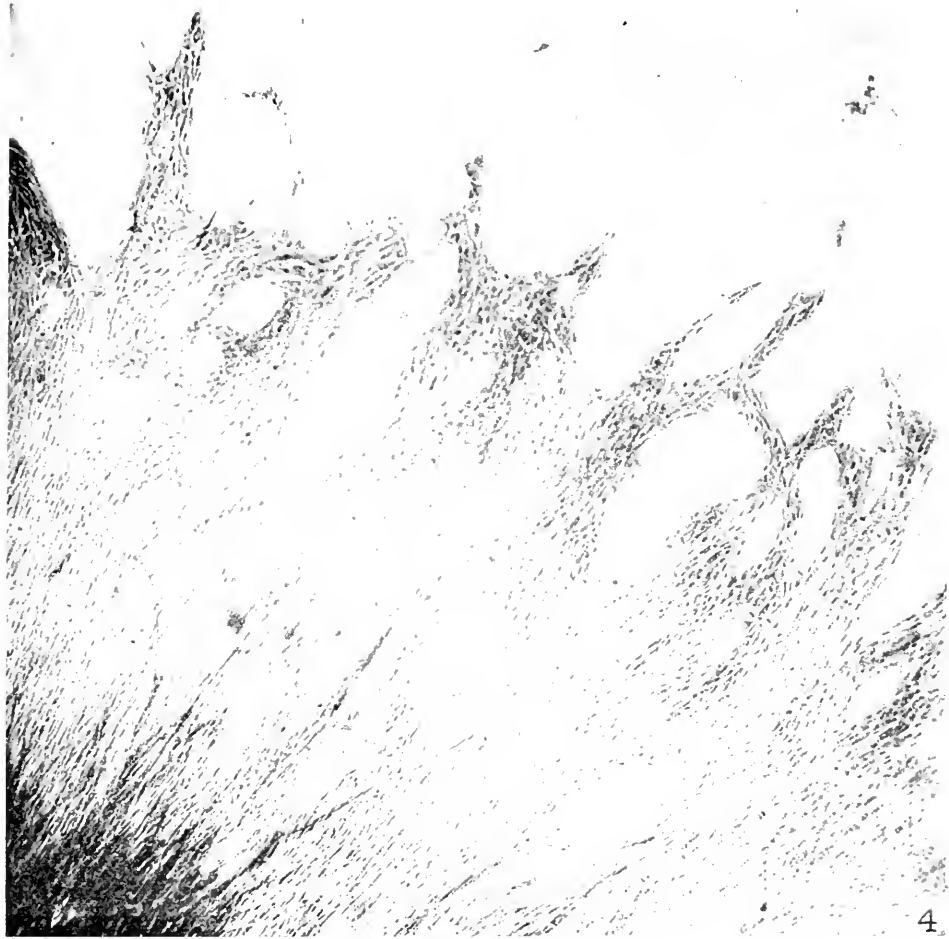


Fig. 16

Experiment 22970—2. 17 passage of a pure culture of epithelium growing in a membrane. Fragment cultivated embedded in the clot. · about 80.
From The Journ. of Exp. Med. 1922, XXXVI, 285.

When we intentionally embed the epithelial tissue in the plasma clot, the growth becomes very irregular to begin

with. After the cultivation of epithelium for a certain length of time embedded in the clot, the growth can suddenly set in with great intensity. The growth is then very regular and extensive. The cells form a membrane, usually consisting of one layer of cells, but the individual cells are no longer polygonal, but spindle-shaped (Fig. 16, 17). Still this picture does not resemble a culture of fibroblasts. The cells are closely in contact with each other; their protoplasm is spread out and gives the appearance of flat cells. In the periphery of such a culture we shall very rarely find a network as in the fibroblasts culture; but we shall see smaller or larger tongue-like processes consisting of many cells in close relation to each other, which practically is never found in the connective tissue cultures. When we have this type of culture, fusiform epithelial cells growing in the clot and producing even a membrane-like growth of new cells all around, we have a type of epithelial cells which is found to grow very rapidly. An illustration of this type can be seen in Fig. 16 and 17.

Besides this rapidly growing type of epithelial cells, we have the one which grows in winding tubules (Fig. 15). This type grows very slowly and irregularly. The tubules or solid processes protruding from the clot may be long and thin or short and thick. These tubules are often very peculiar, that is, they are very slightly refractive, so that they can scarcely be distinguished in the microscope. Sections made on such cultures may show tubules with a distinct lumen; sometimes we have only a single layer of cells around the lumen, and sometimes we can see several layers. At other times we have no tubules, but solid epithelial processes.

Fig. 18 shows such epithelial peninsulas shooting out from the tissue fragment. These look very similar to the solid processes of invading epithelium we know from certain carcinomata at the line of demarkation between carcinomatous and normal tissue. Recently there has been

a discussion about the tubule formation being the result of the action of contaminating fibroblasts (Drew, 1917).

As a whole the epithelial cultures are easily multiplied. Some of them are more difficult to multiply, namely, those



Fig. 17.

Experiment 25970—2. The individual cells appear spindle-shaped and flat.
magnification about 200.

From The Journ. of Exp. Med. 1922, XXXVI, 285.

which form the tubules: they are often small and can hardly be extirpated entirely from the clot. The surface grown epithelium sometimes gives trouble: after extirpation it usually contracts, as does a drop of mucus, and is difficult to replace on the surface of the clot in such a way

that it is spread out. But after a little practice this can be accomplished.

During the cultivation of epithelial cells in vitro, contamination with fibroblasts may very easily occur. Sometimes a small piece of tissue from the embryonic tissue juice is accidentally carried over; sometimes it grows out a little distance from the epithelial tissue, but at other times fibroblasts can be seen growing in the periphery of the epithelium. The cultures have to be examined carefully and if contaminated with fibroblasts they must be kept separately for observation for quite some time. It sometimes happens that the culture of epithelium commences to grow at a suspiciously quick rate and the whole appearance of the culture has changed to a fibroblast-like culture; in such cases we doubtlessly have had a contamination with fibroblasts which has escaped notice. The contamination is mostly found when the epithelium is allowed to grow embedded in the clot. The explanation is quite natural. When the tissue fragment is placed in the fluid plasma and a drop of tissue-juice has been added, the drop is then stirred up with the knife and mixed. If a small piece of tissue from the tissue juice should have escaped our notice and should be brought to the plasma with the juice, it will usually agglutinate to the original tissue fragment, and when coagulation takes place these two pieces will grow together and the error usually is not detected before the connective tissue cells appear everywhere and the culture must be discarded. This has happened rather often; usually the tissue which causes the contamination can scarcely be seen in the juice; it is often a tiny jelly-like substance of practically the same refractivity as the juice and can be found floating in the upper part of the fluid extract.

There are two ways of avoiding this error; either to prepare the embryonic tissue juice by grinding up the embryos with sand or kieselguhr or by using a Buch-

ner's press; there will then be no living tissue cells in the juice. It is also the safest to store the tissue juice in the refrigerator for a couple of days before using it. The other method is to cultivate the epithelium on the free surface of the coagulated plasma-extract medium. In



Fig. 18.

Experiment 1128—2. 13th passage of a pure culture of epithelium growing embedded in the clot. The new growth appears as solid processes and tubules. Stained with Azur II. \times about 160.

From The Journ. of Exp. Med. 1922, XXXVI, 285.

the latter method the plasma and the tissue juice is mixed up and allowed to coagulate before the tissue fragment is brought to the medium. If a small piece of foreign tissue

should happen to appear in the plasma, it is very seldom that it is in just the same spot as the fragment and the contamination can be detected in time. We have been cultivating epithelium in that way and have covered it with a tiny drop of Ringer's solution instead of tissue juice and the number of fibroblast-contaminated cultures was extremely low.

The precautions mentioned here show one thing which had not been realized before or taken into consideration, namely, how often a contamination with foreign cells is possible. It is quite naturally noticed here in the experiments on the cultivation of epithelium for long periods of time. The epithelium grows quite differently from the fibroblasts, and it is therefore very easy to see when connective tissue cells appear in the culture. On the other hand, it is impossible to discover in cultures of fibroblasts when new connective tissue cells accidentally are added to the tissue fragment. Therefore, it is absolutely urgent to take strict precautions in working with pure cultures in order to avoid disastrous errors.

In order to settle the question of dedifferentiation of the epithelial cells in vitro a series of experiments was made in which epithelium and connective tissue were cultivated side by side in the same culture medium. There was always a sufficiently striking morphological difference between the two cultures, the connective tissue forming a typical network of cells, and the epithelium growing in compact masses with the cells in close contact with each other (Fig. 19). These cultures were stained with methylene blue. It was impossible then to see if there was any amalgamation between the epithelial cells and the fibroblasts when the cells of the two cultures were intermingled. Then it was essayed to stain mixed cultures with Van Gieson's stain, but no differential staining resulted from it. In other experiments the two strains: 2-3 months old epithelium and 10 years old connective tissue, were allowed to grow

together for several passages in the same culture. After a few passages the connective tissue cells had overgrown the epithelium and the culture looked like an ordinary fibroblast culture. After about seven passages the culture

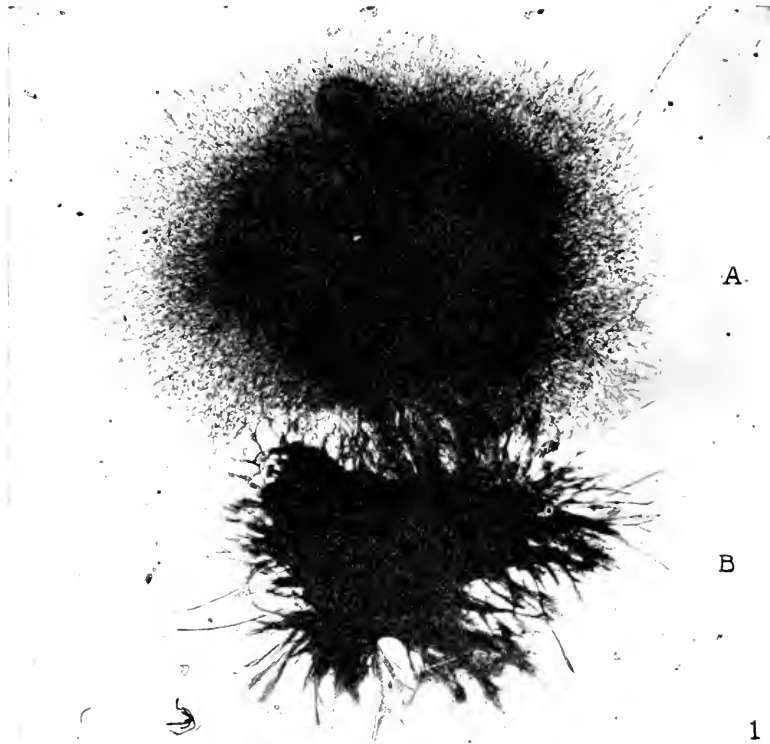


Fig. 19.

Experiment 1532-4. Culture of fibroblasts and epithelial cells cultivated together, after 48 hours incubation. Stained with methylene blue. A, fragment of a 10 year old strain of fibroblasts; B, fragment of a 2 month old strain of epithelium. \times about 20.

From The Journ. of Exp. Med. 1922, XXXVI, 285.

was fixed and sectioned and stained with Van Gieson's stain. The picture which was obtained here was very striking (Fig. 20, 21). In the middle of the dense connective tissue,

which was stained pink, appeared the epithelium stained yellow greenish. In parts of the culture the epithelium had formed a gland-like structure. Several tubules could be seen with distinct lumina surrounded by a regular high epithelium. The nuclei were all located near the basal membrane. Some of the lumina were filled with a colloidal secretion and stained yellow. It is worth noticing that the Van Gieson's stain does not differentiate between epithelium and fibroblast when the culture is stained as a whole, only in sections. The plasma clot seems to prevent the differential staining.

This experiment shows clearly that the two strains of cells remain definite by being cultivated in vitro and it shows also that the epithelium has a definite formative ability. Champy¹²⁷⁾ has stated that epithelium does not dedifferentiate when connective tissue is present. In our experiments we mixed two strains of tissue cells, which had been cultivated in vitro for a long period of time, and should have dedifferentiated a long time ago after the assumption of Champy¹²⁷⁾. The chemical difference of the cytoplasm of epithelium and connective tissue gives us, by using Van Gieson's stain, a beautiful test for the presence of the two cell types.

Now since it is a fact that we do not have any dedifferentiation or transformation of epithelium in vitro, possibilities are opened up for new investigation of the interactions of the two kinds of cells. It would be of the greatest interest first to find out if there is any difference in the most favourable conditions for growth of the two cell types in vitro.

There seems to be one main difference, at least, between the epithelial cells and the connective tissue cells, and that is their behavior to the stroma. The epithelial cells follow the way of least resistance to a greater extent than do the fibroblasts. The individual fibroblasts are able to penetrate through a dense meshwork of fibrin without any difficulty.

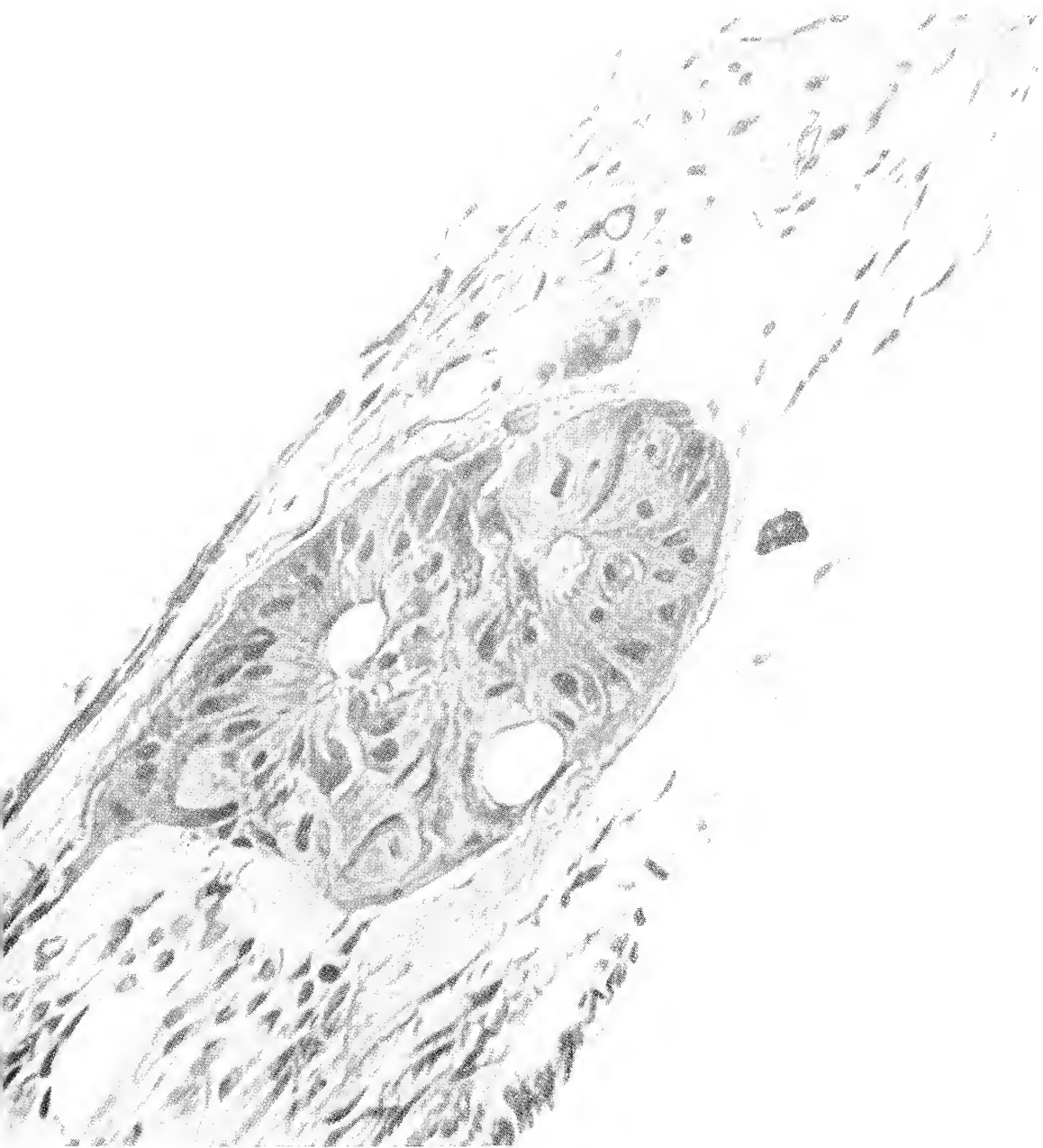


Fig. 20.

The epithelial cells do not possess this ability to such an extent. When the latter grow in vitro they seem to retain their flat cell shapes. They liquefy the culture medium to a great extent. It is often observed how the fragment of epithelial cells makes a concavity in the clot when they

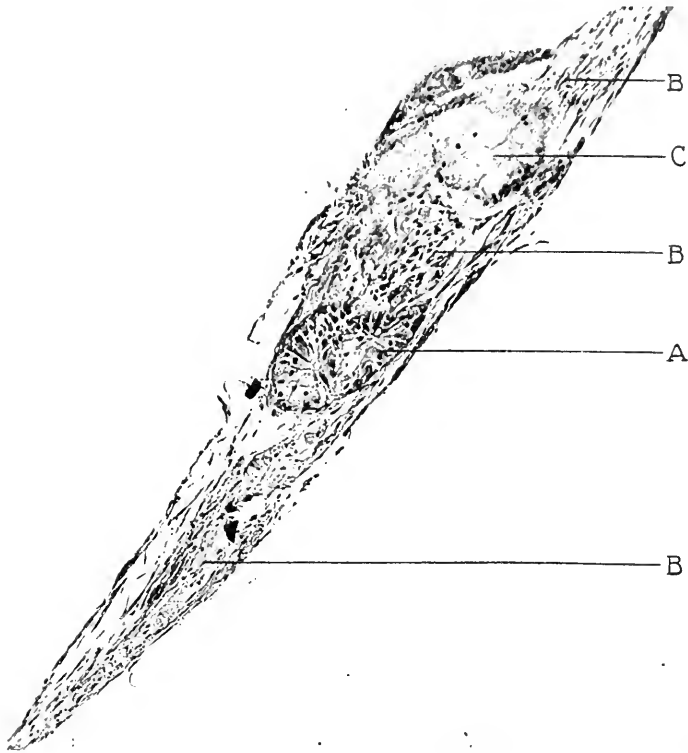


Fig. 21.

Same section as Fig. 20. 120.

From The Journ. of Exp. Med. 1922, XXXVI, 285.

are cultivated on the surface. They simply grow in sheets consisting of cells in one layer and liquefy the medium during the growth so that a large amount of fluid is found covering the culture after forty-eight hours incubation.

The greatest liquefaction takes place at the central portion because of the many cells which are there already at the beginning of the cultivation, therefore the cup-like impression of the culture medium.

The rate of speed at which the epithelial cells grow, compared with that of the fibroblasts, is slightly lower. —

Recently Drew¹⁵⁶ has developed a method by which he is able to get pure cultures of epithelium. By exposing a culture which contains as well epithelium as fibroblasts, to ultraviolet rays, after first having covered the areas of the culture where the epithelial cells are located with droplets of mercury — he is then able to kill the fibroblasts, and the epithelial cells, covered with mercury will remain alive.

Another difference which may be of considerable importance is that the epithelial cells do not change the fibrin fibrillae into structures similar to the connective tissue fibrillae. We know this already. But this fact seems to have a very important bearing upon the physiology of these two tissue types. The epithelial cells seem to have a high proteolytic effect on the fibrin fibrillae. The connective tissue cells have this ability also, in part, of liquefying the medium, but on the other hand, it is able to change the milieu exterieure in such a way that large, strong connective tissue fibres are produced. At the present time there is still a discussion as to the origin of these fibres, whether they are of extra-cellular or intra-cellular origin. The relation of these connective tissue fibrils to the composition of the culture medium is very interesting and will be mentioned in the chapter on controlled or somatic growth of complex tissues.

As a supplement to these experiments, I attempted to isolate and cultivate other kinds of epithelium than the iris, especially epithelium with special functions. Although these experiments are made quite recently and unpublished, I will briefly describe the technique.

A neuro epithelium has been isolated and cultivated for a couple of months in vitro. If we dissect through the posterior wall of the eye bulb, it will be observed that a part of the retina sticks to the corpus vitreum. This part can be cultivated without greater difficulties and is found to grow in cultures in the same typical way as the iris epithelium.

A mucous-secreting epithelium from the small intestine of the chicken embryo I have isolated and cultivated. — A small section of the upper part of the intestine of a chicken embryo 15–17 days old is placed embedded in the ordinary culture medium. — Out from the lumen of the intestine the epithelium grows in big sheets and can easily be separated and transferred into a new culture. It will now be interesting to see, if the mucous-secreting epithelium for instance continues to secrete mucous in vitro and interesting to see if epithelium with pigment is able to produce pigment in vitro.

Innumerable possibilities are opened for the study of the special functions of various tissue cells in vitro under different condition of experiment. It is obvious that the study of the interactions of the various tissues will lead to interesting discoveries.

Quite recently Ebeling personal communication has succeeded in obtaining a pure strain of thyroid epithelium.

CARTILAGE CELLS.

In the literature on tissue cultivation a few scattered notes can be found about cultivation of cartilage in vitro. In 1910 Carrel and Burrows⁷¹ cultivated conjugal cartilage among other tissues from adult animals. At that time no attempt was made to isolate pure cells for cultivation.

In working with the eye of the chicken embryo, I observed in sections from the eye-bulb that it probably would be rather an easy matter to dissect free the thin layer of cartilage in the sclera of the eye. The eye of birds and

fishes has, as is known, often a *pars cartilago sclerae* and sometimes even a *pars ossea*.

The experiment was undertaken in the following way: On the excised eye bulb, the dark pigmented *membrana nictitans* was pushed aside with a small knife and by means of an iris forceps the thin semi transparent membrane, which is the cartilage, was taken out. The eyes used, were taken from chicken-embryos about 15–18 days old. The manipulation was not so easy as it appeared from the beginning. Many pieces of cartilage proved later to contain elements of connective tissue and the cultures were discarded.

The technique for the cultivation was briefly this. The fragments were placed on the free surface of the coagulated plasma-juice mixture and covered with a little Ringer's solution or tissue juice. The composition of the culture medium was the same as that used for fibroblasts and epithelium.

The cartilage did not grow at all when it was embedded in the plasma clot. The fragment was loosened and fell to pieces and proved to be extremely difficult to transfer to a fresh culture medium. The appearance of such a culture of cartilage cells which was cultivated for some time embedded in the clot looked almost like a culture of yeast. There was no cohesion of the individual cells, which were round and extremely small. By examination with high magnification, the cells had much the same appearance as lymphocytes. They were of the size of small lymphocytes with a very sparse cytoplasm and a nucleus which took the stain very intensively (Fig. 22). The stroma disappeared after quite a short time of cultivation and the tissue fell apart. In making stained specimens after varying intervals of incubation, the loosening of the tissue could be followed quite well step by step (Fig. 22). The hyaline substance was loosened and finally disappeared. The cells migrated out or became free of the amorphous substance, but did not produce any new and died.

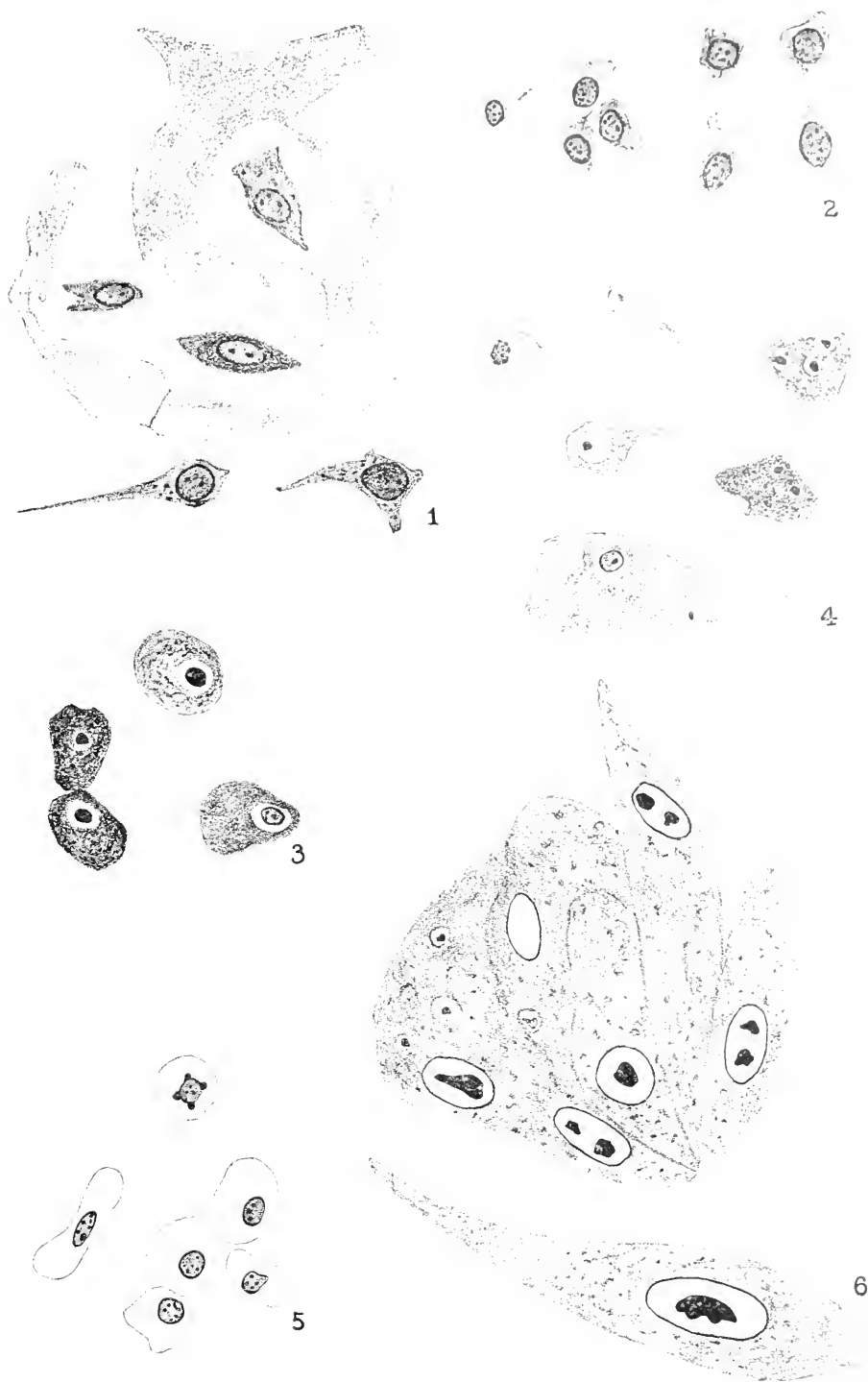


Fig. 22.

- 1) Cartilage after 48 hours incubation in plasma medium. The amorphous substances are broken up. The cartilage cells contain relatively little cytoplasm and the nucleus and cytoplasm are stained very deeply. Stained with azure blue.
- 2) Types of isolated cells of cartilage from 2 day old cultures.
- 3) Types of cartilage cells after escape from the amorphous substances.
- 4) Types of cartilage cells cultivated on the surface of the medium.
- 5) Cell types of cartilage found in cultures of fresh cartilage after 48 hours incubation. Nuclei deeply stained, with the granular chromatin arranged in the periphery. The cytoplasm is unstained and clear as glass.
- 6) Cartilage cells growing on the surface of the medium.

From The Journ. of Exp. Med. 1922, XXXVI, 379.

By placing the fragment of cartilage on the free surface of the clot under a film of fluid, the same loosening of the amorphous substance took place and the lymphocyte-like small cells could be seen migrating out (Fig. 23). These cells began to spread out their cytoplasm and grew finally to big cells, many times larger than the former small types (Fig. 22). The nucleus of the big cells was almost as

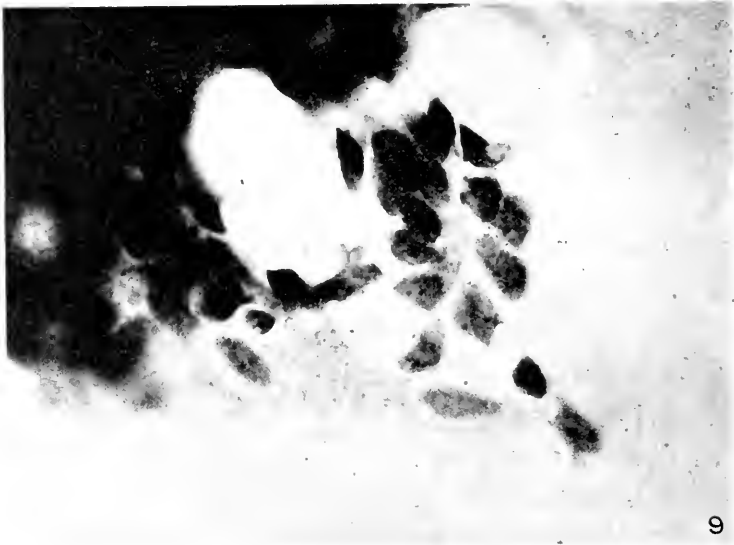


Fig. 23.

Fresh cartilage cultivated on the surface of the plasma clot, showing the small types of cells just migrating on the medium. Stained with azure blue. x 1,000.

From The Journ. of Exp. Med. 1922, XXXVI, 379.

big as the entire cells of the type of the small lymphocyte-like cell. All forms of transition could be studied. The cytoplasm of the big cells usually took up the stain much more intensively than the nucleus. The cytoplasm contained frequently numerous large vacuoles and the nucleus usually one big nucleolus.

It is a very interesting transformation which can be seen to take place here. Renault and Dubrenil¹¹ concluded that the various connective tissues and also cartilage originated from the lymphocytes. This seems, apparently, to have some bearing on my experiments with cartilage cells in vitro. Here we are able to follow the transformation under certain conditions, from a lymphocyte-like small cell type to an entirely different type of cell. It was also found in these experiments that the amorphous substances are not produced under the in vitro condition. Another interesting fact is, that the cartilage cells are unable to grow when embedded in the plasma clot. As soon as the tissue is brought to the surface the cells are able to migrate and proliferate.

The main characteristics of the cultures of cartilage cells are these. The initial growth takes place only on the free surface of the coagulated culture medium. After a good growth has been obtained here, the tissue is able to grow embedded in the clot. The cartilage cells form membranes as epithelial cells do, as well on the surface as when embedded in the clot. Liquefaction of the culture medium occurs to a great extent. The individual cells are spindle-shaped with a rather large spherical nucleus containing usually only one nucleolus. The cytoplasm contains often big vacuoles. In stained preparations the cells appear with a lighter stained nucleus and a darker stained cytoplasm.

The segregation apparatus of Renault when vitally stained with neutral red appears to be similar to that of fibroblasts (Fig. 21).

As a whole, the new growth of cartilage cells in vitro is rather delicate and is often difficult to transfer to new culture media. It takes often quite some time before the growth becomes so abundant that the culture can be divided (Fig. 25).

The studies of this tissue are rather interesting because of the definite changes which take place in vitro and can

be followed step by step. Here we have a tissue, which in the organism, under certain conditions produces a peculiar stroma which, when brought to a culture medium under certain experimental conditions disappears. Another interesting fact is that the tissue is unable to grow when it is embedded in the clot; only on the surface the cells are able to spread out. There is no doubt that this phenomenon is due to the different physical conditions which exist in the bulk and on the surface of the clot.

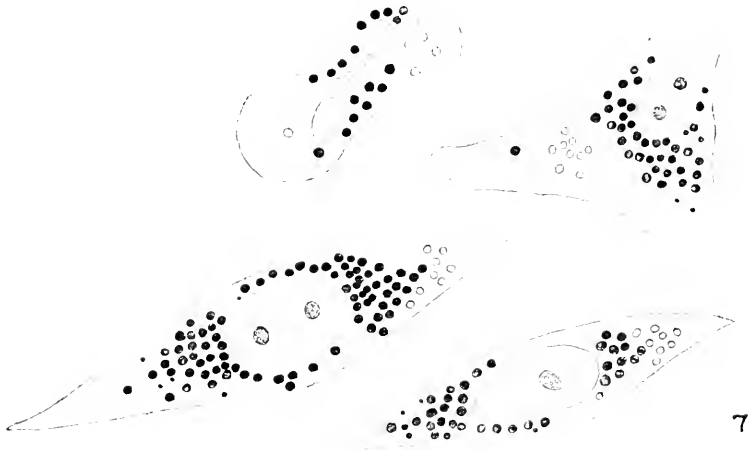
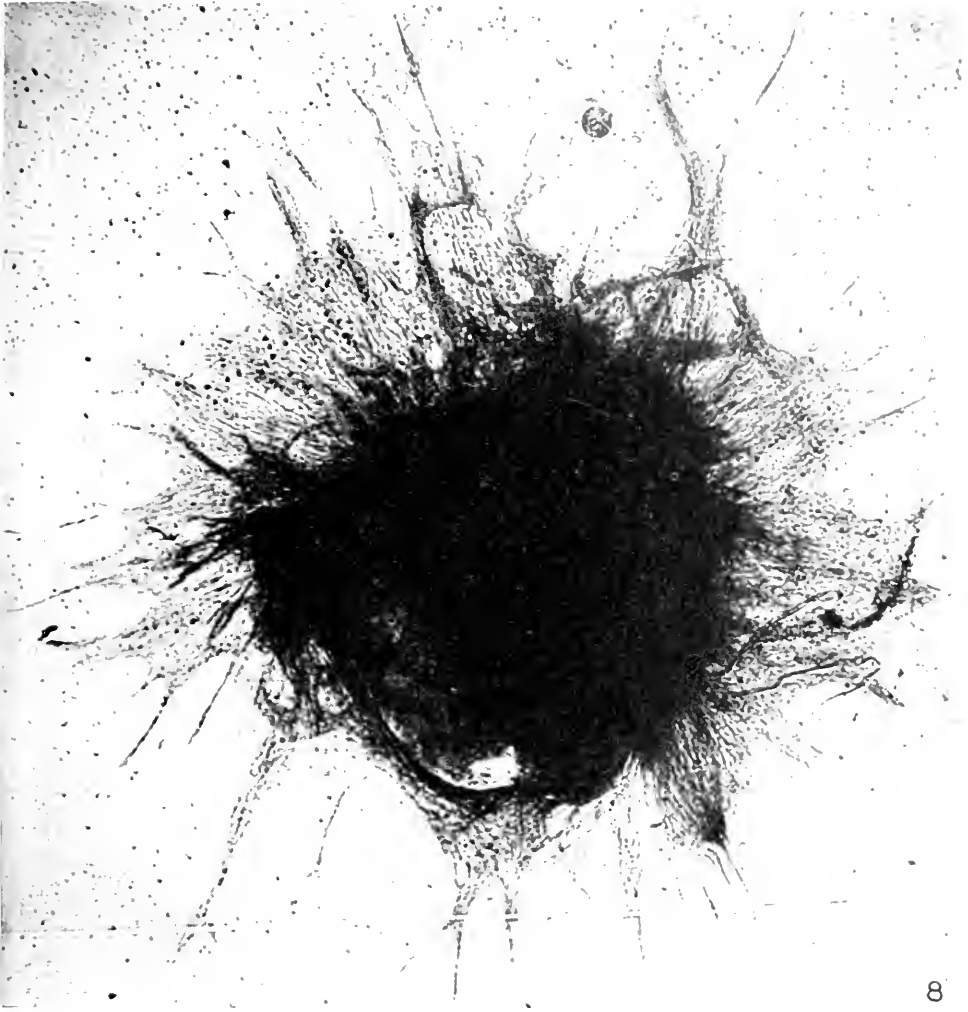


Fig. 24.

The segregation apparatus of Renault, vitally stained with neutral red.
The cultures are 6 Weeks old; 24 hours incubation.
From The Journ. of Exp. Med. 1922, XXXVI, 379.

LARGE MONONUCLEAR LEUCOCYTES.

In 1921 Carrel and Ebeling⁹⁶⁾ succeeded in obtaining a pure culture of large mononuclear leucocytes. The method used here for obtaining the pure cultures is the physiological elective. The method is briefly the following: The white blood corpuscles are obtained by centrifuging at high speed the freshly taken blood from an adult chicken.



8

Fig. 25.

2 month old culture of cartilage cells after 48 hours incubation. Stained with azure blue. x 60.

From The Journ. of Exp. Med. 1922, XXXVI, 379.

After the removal of the plasma, a few drops of diluted embryonic tissue juice is placed on the surface of the coat of leucocytes. About fifteen minutes later, the coagulum containing the white cells can be removed and placed in a watch glass with a small amount of Ringer's solution. Nearly all the red cells can now be washed away and small fragments of this coagulum containing the white cells can be transferred to the culture medium. The medium



10

Fig. 26.

Experiment 1634. Mitotic figures in cartilage cells. Stained with azure blue, x 1,000.

From The Journ. of Exp. Med. 1922, XXXVI, 379.

used for cultivation consisted either of two volumes of plasma and one volume of chicken embryo juice, or of one volume of plasma and one volume of chicken embryo juice, and two volumes of Tyrode solution. Great care must be taken to avoid contamination of the cultures with other cells from the tissue juice. Therefore, the embryonic juice was diluted with Tyrode solution and centrifuged for a long time at high speed.

Small fragments of the coagulum from the blood, containing the white corpuscles were embedded in the plasma. tissue juice was added and coagulation then takes place. It is important to know that if there are too few leucocytes in the culture or if there are too many, they die. After forty-eight hours incubation, or sometimes even longer, 1—5 days, the original fragment was cut out, washed in Ringer's solution and placed in a fresh culture medium. Here it is very important to cut the culture out of the



Fig. 27.

24 hour culture of leucocytes taken from the blood. In the upper left corner is seen part of the original mass of cells. x 62.

From Carrel and Ebeling: Large mononuclear leucocytes. The Journ. of Exp. Med. 1922, XXXVI, 365.

clot with clean cuts. If the edges are crushed or folded, no migration takes place.

If the cultures were made in the proper way, the cells could be seen to migrate into the medium after twenty-four hours. The outer zone was formed of small amoeboid cells, which in stained preparations proved to be polymorphonuclear leucocytes. Fig 27. The inner zone con-

sisted of larger ameboid cells, some with large reticular pseudopods and others with small filiform or lobar pseudopods. After a few passages the polymorphonuclear leucocytes disappeared and a little later also the small lymphocytes. The cells which remained and continued to multiply were the large mononuclear cells. The general appearance of the culture was very different from a culture of connective tissue cells. The cells had no tendency

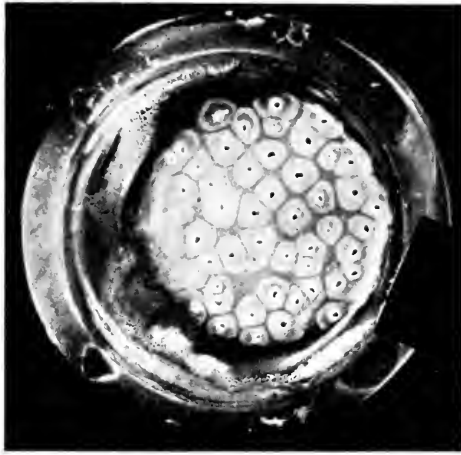


Fig. 28.

Colonies of leucocytes in a Gabritschewski dish, showing their lack of coalescence.

From Carrel and Ebeling: Large mononuclear leucocytes. *The Journ. of Exp. Med.* 1922, XXXVI, 365.

to form a tissue, but remained isolated. When colonies of leucocytes were grown together in a Gabritschewski dish, the colonies showed no tendency to unite as colonies of fibroblasts or epithelium always do (Fig. 28).

The large mononuclear cells which migrated into the new medium were elongated or branched. The anterior end of the cells showed usually very active pseudopods. When the cells were stained the nucleus become dark and

the cytoplasm clear. Sometimes interesting morphological changes occurred. The pseudopods disappeared and the outlines of the cells became very sharp. The cells looked perfectly like fibroblasts and they were liable to form anastomotic processes with other similarly differentiated cells (Fig. 29). Often a long chain of cells could be observed and even a chain would show the transition forms from the ameboid cell in the distal part of the chain to the perfect fibro-



Fig. 29.

Culture of a pure strain of mononuclear leucocytes undergoing differentiation.
Eighth passage.
From Carrel and Ebeling: Large mononuclear leucocytes. *The Journ. of Exp. Med.* 1922, XXXVI, 365.

blasts in the central part of the chain. Also in the stained preparation the similarity to the fibroblast was very striking. The outlines of the cells were very sharp, both ends drawn out to a minute process, the nucleus very clear containing two nucleoli.

Experiments were undertaken by Carrel and Ebeling⁹⁶⁾ to see how the lymphocytes respond to different amounts of embryonic tissue juice in the culture medium.

The result was that the lymphocytes were much more sensitive to the tissue juice than the fibroblasts. The optimum condition was found to be about 30 per cent tissue juice.

These observations are of great importance and may probably partly settle the question of the origin of fibroblasts from large mononuclear lymphocytes during inflammatory conditions, as has been discussed from the days of Virchow and Cohnheim.

The action of homologous serum on the growth of lymphocytes was also studied and it was found that the cells were very sensitive to the inhibiting influence of the serum.

This method of obtaining pure cultures of large mononuclear leucocytes is a physiological elective, because all other types of white blood corpuscles disappear spontaneously when cultivated under the said experimental conditions. First the polymorphonuclear leucocytes disappear and a few weeks later also the small mononuclear cells. It was possible to retain the large mononuclear leucocytes in the cultures for nearly three months. The rate of multiplication was much slower than that of fibroblasts and epithelium. An interesting fact is that the cultures of these cells were much more sensitive to all influences, whether of physical or chemical nature. And still the most interesting and important result from these investigations is the transformation of the large mononuclear leucocytes into tissue-forming cells of the type of fibroblasts. Not only it is interesting to observe the perfect morphological change these cells may undergo, but more important, it seems to me, is the change which can take place to tissue-forming cells from a type of cell which under certain conditions absolutely does not show any tendency to form anastomosis. Maximoff ¹¹⁰ has probably observed the beginning of the same transformation.

For a long time it has been assumed that the lymphocytes are the origin of the various types of connective tissue. Renaut and Dubreuil ¹¹⁵ were of that opinion and the observations of Maximoff ¹¹⁰, Carrel and Ebeling

⁹⁶⁾ and Fischer ¹⁹⁴⁾ seem to confirm it. The conditions under which the differentiation takes place in vitro are not yet known. It does not occur in all cultures of large mononuclear leucocytes, but mainly in those which grow very slowly.

VI.

TISSUE CULTURE AS A PHYSIOLOGICAL AND ANATOMICAL METHOD.

1. THE HUMORS OF THE BODY.

The strains of fibroblasts and epithelium grow with an astonishing regularity, and are found to respond promptly to changes in the composition of the culture medium by a modification of their rate of multiplication. The strains can therefore be used as reagent for the detection of substances contained in the humors which have the power of activating or decreasing the rate of cell proliferation.

From the very beginning of the researches on explanted tissue cells, Carrel⁵⁷ observed a constant relation between the rate of growth and the composition of the medium. A definite programme for investigation was followed by Carrel and his associates. Cell phenomena such as multiplication, growth and senility were studied. It was stated by Carrel that from the time of Claude Bernard it was known that the life of an organism is the result of the interactions of the cells of which it is composed and of their milieu interieure. In order to discover the laws by which the cell growth is regulated it would be necessary to modify the humors of the organism and to study the effect of these modifications on the growth of the tissues.

The works of Carrel and his pupils of the last 15 years is mainly characterized as a study of the humors of the body with strains of tissue cells as reagents. The work is ingenious in its continuity and prospectivity. The results are of the greatest importance and dominated by many new points of view.

It was assumed rather early by Carrel⁵³, that a medium more efficient than normal plasma could be found, as

the tissues in the organism certainly did not meet with the best possible conditions of growth. If the blood was the best possible medium, the cells would grow without restraint, the organs and tissues would lose their relative size and morphology, and the whole body would become monstrous.

The variations of growth of certain tissues were then studied in plasma, the conditions of which had been modified.

It was discovered by Loebl, in the growth of certain organisms that a slight alkalinity or hypotonicity increased their growth considerably. The same sensitiveness was observed by Carrel for living tissue cells in vitro. Modifications of the osmotic tension or the alkalinity, or the addition of certain inorganic salts to normal plasma, increase the rate of growth of tissues. Ebeling¹⁶⁰ found that an increase or decrease in the osmotic tension of the culture medium at first stimulates cell proliferation, but eventually retards it and proves to be unfavourable to growth. Hogue²⁶⁰ investigated the influence of various osmotic tensions qualitatively on the tissue cells.

In the fundamental experiments of J. Loebl on artificial parthenogenesis, it was demonstrated that cell division can be induced by slight changes in the composition of the sea-water in which the sea-urchin's eggs are placed. It was therefore supposed by Carrel⁵⁸ that certain modifications of the milieu interieure of the tissues of mammals would bring about the multiplication of their cells. Already in 1907—08 Carrel studied the processes of reparation of small cutaneous wounds and the action of various substances on the rate of cicatrization. It was observed that the proliferation of epithelium and of connective tissues was activated by dressing the wound with the pulp of tissues and organs. Pulp of thyroid gland deposited on wounds of the dog brought about the formation of exuberant granulations. Applied to bones, it produced a marked thickening of the periosteum.

The influence of extracts from various tissues was tried on the isolated tissue cells *in vitro*. Extracts of chicken embryos, of spleen, kidney, muscles, Rous sarcoma, thyroid gland were made and added to the plasma medium of the tissue cells and the extent of growth measured. The results obtained at that time, when the technique for tissue cultivation was not as perfect as it is now, were rather striking. All the tissue extracts were found to activate growth of the connective tissue, but the degree of activation varied much. Embryonic tissue juice was the most active. Extracts of adult spleen and the Rous sarcoma were almost as active as the extract of chicken embryos. Kidney and heart extracts were much less active, while the extracts of connective tissue and of blood corpuscles brought about a slight acceleration only of the growth. The influence of the thyroid gland extract and the muscle of the dog on the growth of periosteum was very marked. Thyroid extract was more active than muscle extract. It was also observed that when the extracts were heated to 56° C. for from 10 minutes to half an hour they almost lost their activity. Filtration of the extracts through Berkefeld filter, caused a considerable decrease in the activity of them, and filtration through a Chamberland filter suppressed completely the activating power of an extract. This has already been mentioned in the chapter on the culture media.

It was learned as a fact, that the tissue cells are able to live for a short time in plasma alone; and that the death of the tissues could be prevented by adding small amounts of extracts from chicken embryos. The plasma did not contain the substances necessary for the indefinite multiplication of fibroblasts. Although the fibroblasts cannot live indefinitely in plasma, they often grow with a great activity for several weeks. According to Lewis³⁵⁰, Ingebrigtsen²⁶⁹ and Burrows³¹⁻⁴⁰ the tissues grow at the expense of the food stored up in the bodies of the cells. Therefore an actual increase of the bulk of the tissue was

never observed, it is a mere survival of tissues. The addition of embryonic tissue juice to the plasma activates the cell-division and brings about an immense increase in mass of the tissue. The strain of fibroblasts of Carrel derived from a small fragment of embryonic heart has produced about 30,000 cultures in 9 years and is as active to-day as at the beginning of its life. Carrel and Ebeling⁹² found that connective tissue grew as extensively in fibrin fixed by formaldehyde as in normal fibrin, therefore the rôle of fibrin must be considered as purely mechanical.

The influence of serum was investigated by Carrel and Ebeling⁹³ by measuring the rate of growth of fibroblasts in media containing no serum. The fibroblasts were cultivated in media composed of fibrinogen suspensions and of Tyrode solution and containing no serum or serum in varied dilutions. The fibrinogen was prepared according to a technique described by Ebeling¹⁶². The results of the growth of fresh embryonic heart tissue in media containing from 0 to 90 per cent serum showed that the cells in their process of multiplication do not make use of the serum to an extent measurable by the present technique. The growth of embryonic heart appeared to be independent of the concentration of the serum as long as the amount of fibrin was not modified. Also the old strain of fibroblasts was cultivated in media containing serum from 2.37 to 80 per cent; there was not observed any difference between the amount of growth of the fragments; the rate of growth decreased rapidly and death occurred usually after the 6th or 7th passage.

When fresh embryonic heart tissue and connective tissue from the old strain were cultivated in serum and fibrinogen only, there was a striking difference between the growth of the two fragments. As soon as the strain of fibroblasts was placed in plasma alone, the rate of multiplication of its cells decreases, while under the same conditions the activity of a fragment of embryonic heart increased for

several days. The life of heart tissue was much longer than that of the strain of fibroblasts. This phenomenon is explained by the fresh embryonic heart containing these accelerating substances as they are found in the embryonic juice—whereas the old strain of fibroblasts contains very little compared with the fresh tissue.

In order to learn whether the embryonic tissue juice, which causes the indefinite multiplication of fibroblasts *in vitro*, renders possible the utilization by the cells of substances contained in the serum or the fibrin—or whether it supplies itself the material required by the cells for their proliferation, Carrel and Ebeling³³ made the following experiments.

In some experiments the media were composed of a constant amount of fibrinogen suspension and embryonic tissue juice and varied quantities of serum and Tyrode solution. The results of these experiments showed that the lack of serum, or its presence in low or high concentrations, had no influence on the rate of growth. This demonstrated that in the presence of embryonic tissue juice, there was no utilization of the serum or fibrin to a measureable extent.

In other experiments the embryonic tissue juice was added in increasing amounts to a constant amount of serum and fibrinogen. The results of these experiments were that increases in concentration from 0 to 10 per cent of embryonic juice brought about a greater change in activity of the cells than increases from 10–80 %. After the concentration of the juice in the medium had reached 10 per cent, no further increase was observed at any higher concentration. (Fig 30.)

Already in the early period of tissue cultivation Carrel³⁷ discovered that by cultivating connective tissue in the plasma of chickens of different ages, he found that the growth was more abundant in the plasma of the younger than in that of the older animals. This fact suggested that

growing fibroblasts could be used as a reagent for the changes occurring in the blood under the influence of age. Loeb and Northrop³⁶⁸ came to the conclusion of their experiments on the temperature coefficient of duration of life of the fruitfly *Drosophila*, that the duration of life was probably determined by the production of a substance leading to old age, or by the destruction of a substance normally preventing old age. In order to know

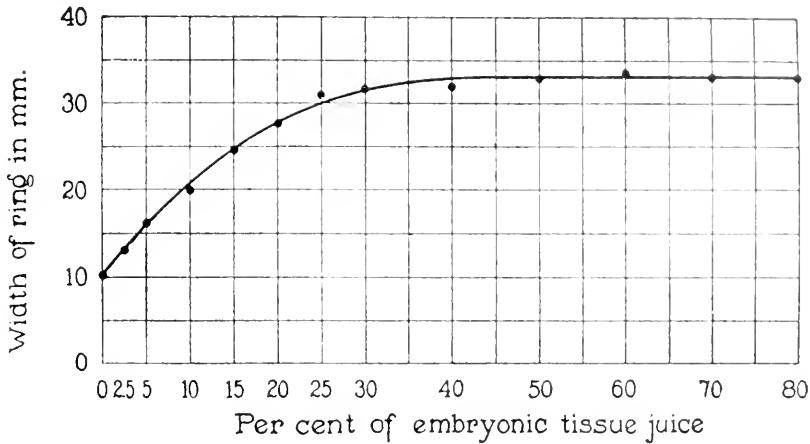


Fig. 30.

Relation between absolute increase of the tissue and the concentration of the embryonic juice in the medium.

From Carrel and Ebeling: Multiplication of fibroblasts in vitro, Journ. of Exp. Med. 1921, XXXIV, 333.

which of these hypotheses was the right one, Carrel and Ebeling³² made a series of experiments on fibroblasts cultivated in plasma from chicken representing different ages.

It was observed that the growth of fibroblasts in plasma from young chickens was more abundant than that in the plasma from the old chickens³². When a fragment of connective tissue from the old strain was placed in plasma alone, a sudden drop in the velocity of growth was ob-

served. This resulted from the suppression in the medium of the active substances contained in embryonic juice. If the growth was compared of two such fragments in plasma alone, the one cultivated in plasma from a young chicken and the other in plasma from an old chicken, it was observed that the growth and proliferation stopped entirely and the death occurred much sooner of the fragment cul-

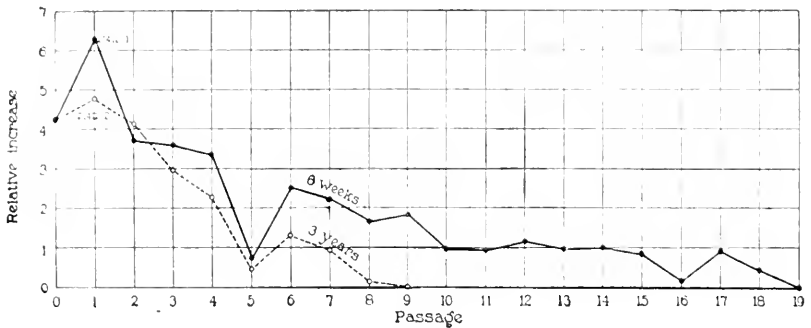


Fig. 31.

Comparison of the rate of growth of fibroblasts in the plasma of a 6 week old chicken and a 3 year old hen. The sudden drop in the rate of growth of the culture in the 6 week plasma was accidental. The small amount of growth obtained at the 5th passage was due to the transfer of the tissues to a fresh medium after 24 hours instead of 48 hours.

From Carrel and Ebeling: Age and multiplication of fibroblasts. *Journ. of Exp. Med.*, 1921, XXXIV, 602.

tivated in the plasma of the old chicken, than of the fragment in the plasma of the young animal Fig. 31. In many cases the fragment cultivated in plasma from a 3 months old chicken died after 11 passages, while the fragment cultivated in plasma of a 3 years old chicken died after 7 passages. In another experiment a fragment cultivated in plasma of a 3 months old chicken died after 16 passages, while a fragment cultivated in plasma from a 9 years old chicken died after 3 passages. The duration of life of the cultures in the plasma of the old animal was

only 19 per cent of that in the plasma of the young animal. The result of these experiments was that the rate of multiplication of fibroblasts and the duration of their life in vitro varied in inverse ratio to the age of the animals from which the plasma or the serum was taken.

Experiments were undertaken by Carrel and Ebeling⁹², to examine if the action on the growth of fibroblasts of serum, taken from animals of advanced age could be explained by the disappearance from the blood of an accelerating factor, or the development in the blood of an inhibiting factor. The working hypothesis of the investigators was, that if youth were supposed to be characterized by a factor present in the blood and activating the multiplication of fibroblasts, and senescence by a decrease in the power of this factor, connective tissue cells should proliferate more actively in a medium containing serum under a high concentration than in a medium with a lower concentration of the same serum. On the contrary, if the same phenomena were caused by the progressive increase in an inhibiting factor, the fibroblasts should multiply more actively in a low than in a high serum concentration.

It was found that the difference in the rate of growth in the low and high concentration of serum or plasma from young and old animals, were practically the same. This demonstrates that the blood serum in growing animals does not contain any accelerating factor for the proliferation of the fibroblasts. Age does not therefore bring about the disappearance from the blood serum of an accelerating factor, but produces the increase of an inhibiting factor for the growth of fibroblasts.

A study of the influence of heterogenic sera, taken from animals of different ages, on the rate of growth of fibroblasts has also been made by Carrel and Ebeling⁹¹. It was found earlier by Carrel, that the amount of growth

in heterogenic serum obtained from young animals was much greater than in heterogenic serum from older animals. It was therefore assumed that chicken fibroblasts could be used also as a reagent for the detection of the modifications brought about by age in the blood of other animals.

If the concentration of the heterogenic sera was increased beyond a certain grade, the inhibiting action of the medium rapidly became marked and often no growth was observed when the concentration reached 30-45 per cent. Sera were obtained from cats and dogs. The inhibiting influence of heterogenic serum was found to vary in direct ratio to the age of the animal from which it was obtained.

Many attempts have been made by Carrel to find some physical or chemical properties of the various sera from young and old animals which would correspond to their biological properties of increasing or decreasing the rate of growth of fibroblasts. From this point of view I have together with Carrel^{*)} made quantitative chemical investigations of the various sera and at the same time their action on strains of fibroblasts was tested.

The relative amounts of globulins and albumins were measured by means of the Robertson's refractometric method. At the same time the amount of total nitrogen and the amino acids and amino nitrogen Sørensen, Van Slyke was determined of the various proteins.

The results of these experiments were, that with the increasing age of the animal, the nitrogenous substances increased, also the inhibiting effect of the sera and vice versa.

It was found recently by Carrel and Ebeling²⁸ that the action of serum on homologous fibroblasts was not due to one inhibiting substance only, but to the combined action of two substances, one activating and the other inhibiting.

^{*)} Unpublished experiments.

In precipitating the serum with CO_2 , it was found that these substances, globulins, increased the activity of fibroblasts, which indicates that a growth-promoting substance had been obtained from the serum. The serum deprived of its globulins showed to have an increased inhibiting effect on the fibroblasts. It was therefore concluded that the restraining effect of serum on the activity of homologous fibroblasts is due in part to the antagonistic action of growth-activating and inhibiting substances.

The growth-promoting principles of the serum, which is precipitated by CO_2 is found by Carrel and Ebeling⁹⁵⁾ to be as unstable as alexin and certain tissue juices, which have the property of increasing the rate of cell proliferation. The alexin and the growth-promoting substances contained in embryonic tissue juice possess the same property of being destroyed by heat and by shaking. It was found that the inhibiting action of shaken homologous serum on fibroblasts was at least 30 per cent greater than that of unshaken serum.

When heterologous serum was shaken, dog serum being used, its inhibiting action on chicken fibroblasts was very much decreased. The hemolytic action of dog serum towards the red blood corpuscles of chicken, was partly inactivated by shaking. The shaking could be compared to the influence of heat. Shaken serum was found always to inhibit the activity of homologous fibroblasts much more than normal unshaken serum. When the alexin of chicken serum was completely inactivated by shaking, its restraining power on chicken fibroblasts became also more marked. On the contrary, dog serum partly inactivated by shaking, was much less toxic for chicken fibroblasts than normal serum. It was assumed by Carrel and Ebeling⁹⁵⁾ that the restraining effect of shaken serum on foreign fibroblasts may be attributed to the destruction of alexin.

After Carrel⁶⁶⁾ had introduced his new technique of cultivating tissues in flasks uninterrupted for 2-3 weeks

instead of 2 days as in the hanging drop method, many problems within the physiology of the tissues can be investigated under much better conditions. It was found that the growth of the tissues proceed at a constant rate for 2–3 weeks and the increase of the tissues is expressed in a parabola when the medium is a nutrient. In a protective medium the growth is expressed by an S-shaped curve. The S-shaped curve indicates the residual activity of a tissue. The activity of a tissue at a given instant, is a function of at least three independent variables, the inherent cell activity, and the concentration of growth-activating and growth-inhibiting substances in the medium. The inherent activity of a tissue is its activity in a protective medium without nutrient material.

This inherent growth energy of tissues has been the subject for special investigations by Carrel⁶⁷. There is a definite relation between the growth energy of a given tissue and its age, and it is found by earlier studies of Carrel that the healing of an aseptic wound is a function of the age of the patient, which is determined by the size of the wound and its index of cicatrization, (du Noüy¹²⁰).

Pure strains of fibroblasts or heart tissue were according to Carrel, placed in the culture flask of the D type and the culture medium was composed of a solid phase 0.5 cc. of plasma and 1½ cc. of Tyrode solution containing 5 % embryonic tissue juice and the liquid phase of Tyrode solution only. The residual life of a fragment of heart of an 8 days embryo varied from 7–12 days. The residual life of a fragment of an 11 year old strain of fibroblasts, lasted 7–8 days. In comparing the residual growth energy of a heart fragment from a 10 days old chicken embryo and a 17 days old embryo, the growth of the older tissue was about 30 per cent less extensive than that of the younger one.

By the method of cultivating tissues in flasks uninterruptedly for long periods of time, Carrel and Ebeling

⁵⁹ found that the tissue cells cannot obtain the nitrogen necessary for their proliferation from chicken bouillon, egg white, egg yolk or pure egg albumin. It was also observed²⁴ that the addition to a protective medium of extracts of tissues from adult animals, stimulated the rate of growth of homologous fibroblasts. Also the addition of heterologous extracts or tissue juice stimulated a culture of fibroblasts to increase in mass, at least for a time. It was shown that mouse embryo juice was just as active on chicken fibroblasts as the embryonic chicken juice. After 26 passages, the rate of growth of chicken fibroblasts was still practically the same in both substances. Rabbit embryo juice, and guinea pig embryo juice showed almost as active on chicken fibroblasts as the chicken embryo juice.

Fibroblasts and epithelium are not able to multiply with blood serum as the only nutrient. It is known that tissues of an adult animal, even an old animal, are able to grow and multiply under the formation of a large amount of new tissues, for instance when wounds are healing. Under the process of cicatrization of a wound in an animal of old age, substances which stimulate the growth must be liberated. Carrel⁶⁰ found that leucocytes supply the adult tissues with substances which activate the rate of proliferation in the same way as embryonic juice. The leucocytes remain during the entire life of the organism, in the embryonic stage of development and contain growth-promoting substances. These substances could be extracted from leucocytes. Carrel⁶¹ also showed that living leucocytes during their life in vitro, secrete growth-activating substances. He also found that leucocytes are capable of setting free growth-activating substances in vivo. In making an aseptic inflammation, small sponges soaked with dilute hydrochloric acid, were placed beneath the skin of chickens. The inflamed tissue was extracted with a small amount of Ringer solution and tested against fibroblasts in cultures. The experiment showed that the inflamed tissue, when quantities of leucocytes have ac-

cumulated, contained substances capable of increasing the rate of multiplication of fibroblasts. Similar experiments were undertaken with injections of staphylococcus suspension, dog red blood corpuscles, turpentine or bouillon into the peritoneum of chickens. The growth of fibroblasts with addition of extracts from these inflamed tissues, proved in many experiments to be 100 per cent greater than that obtained in Ringer solution.

Recently Carrel and Ebeling⁶⁸ have proved that the lymphocytes are capable of transforming some of the serum constituents into the more complex compounds necessary for the nutrition of the fibroblasts and epithelial cells.

Lymphocytes were cultivated on chicken plasma alone in the D flasks; after a month's cultivation, fragments of the plasma were fixed, stained and examined microscopically. It was ascertained that the lymphocytes were in full activity and had multiplied and invaded the entire culture medium.

In another interesting experiment of Carrel and Ebeling⁶⁸ a fragment of spleen was placed at a short distance from a culture of fibroblasts; the rate of multiplication of the fibroblasts was markedly increased, owing to the secretion of growth-stimulating substances by the leucocytes. It was found also that leucocytes do not live more than 10 days in Tyrode solution. This denotes that in a non-nutrient medium, they exhausted their residual energy from 7–10 days. Thus follows that, if leucocytes are cultivated in blood serum for more than 10 days and are able to increase in migration and multiplication of fibroblasts, the material used by them in the elaboration of the growth-promoting substances must come from the serum.

The experiment was undertaken in this way. In a D flask, a fragment of spleen was placed 12 mm. from a fragment of a pure culture of fibroblasts. The liquid part of the culture medium was composed of pure serum, therefore the fibroblasts stopped their proliferation after 5 days. The leu-

coocytes from the spleen fragment slowly invaded the medium and reached the neighbourhood of the colony of fibroblasts. On the 9th day, the fibroblasts had become very large and their cytoplasm was dark and loaded with fat granules. The lymphocytes proliferated very actively and some of them reached the fibroblasts. During the following days, the appearance of the fibroblasts changed. They became slender and elongated and their cytoplasm decreased in volume and they began to proliferate actively, became refreshed and fully active. — This is a beautiful demonstration of the growth-promoting function of the leucocytes and a clean cut experiment proving that leucocytes are capable of transforming the serum into complex substances which supply fibroblasts with nutrient material for their multiplication, substances which are called *trephones*.

2. THE PHYSIOLOGICAL INTEGRITY OF TISSUE CELLS.

It has been much discussed whether the tissue cells have to be considered as genetical and morphological units of the organism, or if certain cell colonies or tissues or the entire organism may be considered as the unit.

The synzytium theory of the organism on the one side represented by Rhode¹⁵⁰⁾, and the bioblast theory Altmann on the other are the extremes of the numerous theories. The "Zellenstaat" or "Bausteintheorie" Virchow, Haeckel is the conception of the perfect discontinuity and independency of the individual tissue cells. On the contrary the "Synzytium" theory or symblast theory, is the notion of perfect continuity of the plasma of the organism by the means of cytoplasm bridges between the cells. These bridges or anastomoses, called "plasmodesms", should serve as itineraries for the transport of alimentary products, of internal secretions and of living cytoplasm.

The technique of making tissue cultures was supposed to solve this question. Steps towards the solution of the question by this technique have not been made so far.

Most investigators are prone to approach the problem of the tissue culture too exclusively from a bacteriological point of view. People use the terms "pure culture" of tissue cells in the same meaning as they speak of pure cultures of bacteria or paramecia. People nowadays are most inclined to consider the tissue cells in vitro as cultures of independent cell individuals.

Long ago I planned experiments endeavouring to solve this problem. The first thing which had to be done was to see if it was possible to obtain growth and multiplication of one single isolated tissue cell in vitro; in other words to see if it was possible to obtain a colony of pure fibroblasts from one single fibroblast, as colonies of bacteria can be obtained from one single bacterium.

In order to get a suspension of living, uninjured tissue cells for the experiments, it was necessary to develop special methods. In the plasma culture, the tissue cells are connected by means of the fibrin threads in the clot and furthermore the cells are intimately adherent to one another by protoplasmatic anastomoses.

A method for isolating single individuals of fibroblasts has been worked out by Roux and Jones⁴⁵⁷. The plasma cultures of fibroblasts were digested by means of trypsin and a suspension of tissue cells was obtained. This method did not prove to be suitable for the purpose as a large percentage was killed and several cells remained connected to one another as cell colonies.

A new technique was therefore devised. The fibrin thread connections were eliminated by cultivating the fibroblasts in a fluid drop of embryonic tissue juice with the addition of extremely fine cut cotton threads serving as a support for the cell growth, and substituting the fibrin mesh-work.

Ordinary absorbent cotton was finely cut between two fingers by means of a small pair of accurate scissors into a fine powder-like substance and sterilized by dry heat. It was suggested beforehand, that if it were possible to cut the cotton threads extremely fine, they might be easily distributed in the fluid medium and each cotton thread being so small that there would be room only for allowing one or two cells to adhere to it; it would therefore be possible to aspirate the liquid drop of culture medium, containing the cotton threads and fibroblasts by means of a fine pipette and afterwards distribute it in a drop of plasma, which after coagulation has taken place, would fix the cotton threads with the few tissue cells on them, in various places within the clot. The method was found to be very satisfactory. After the cells were transferred to the plasma clot, in the above way, they were found to be well isolated and distributed in the plasma and the life of the cells could be observed from day to day.

Soon after the cells had been transferred to the fixed culture medium, they were found to have assumed a spherical form, the cells in the liquid culture which eluded the pipette had also become spherical, a phenomenon which is due to the sudden deprivation of their solid support. This same phenomenon has already been described also by Rous⁴⁵⁷ and Uhlenhuth⁴⁵⁸. The cells looked very much like leucocytes.

One will now observe a lively ameboid movement going on in the spherical fibroblasts in the fixed medium. Often the protoplasmic currents may develop a remarkable turn of speed. — After a few hours, as a rule, the cells will have assumed a typical spindle shape, characteristic of fibroblasts. When this change has taken place all movement appears to cease. In this conditions the cells remain apparently unchanged in outline, but the protoplasm filling up gradually with vacuoles and fat granules, death takes place, and the protoplasm decays and dissipates.

It has not been possible to observe the division and proliferation of an isolated cell, though hundreds of cells have been studied with this object in view. Growth by proliferation has been observed only when a number of cells were in close contact in a culture. These observations lead to the question as to whether intercellular contact is essential to the cells for their growth and multiplication, and what significance the contact has in growth and multiplication of the cells.

It would be interesting to discover, as a supplementary experiment to the above, how excised pieces of different location within the tissue culture, behave in respect to their ability to multiply when transplanted to a fresh medium. For this reason experiments were undertaken to demonstrate how the different parts of a culture grew and multiplied.

Before discussing the problem, it will be well to describe the other methods by which the attempt was made to determine whether isolated cells are able to multiply. The results of the experiments just mentioned may have been due to a technical error. Anyway it has to be taken in consideration that it is not impossible that the manipulations may injure the cells, despite signs which seem to indicate perfect vitality.

When ordinary cultures were cut in two halves and transferred to a fresh medium, it was frequently noted that the cells began to grow out first from the centre of the cut edge of the culture, that is to say from the point at which the cells were most crowded, and only a little later on from the periphery. This has also been noted by Carrel*. From the observation it might be deduced that the central part of the culture has a greater ability to proliferate than the peripheral part, where the cells are more scattered. Some experiments were undertaken to see how minute the fragments of a piece of tissue from a culture could be and retain the ability to multiply when transplanted to a fa-

*) Carrel, A., Personal communication.

avourable culture medium. It was found that if the transplanted piece consisted of a few scattered cells no growth took place, and the cells took on the aspects of degeneration at a time when the control culture seemed to be in perfect condition. By contrast, when the small clumps of cells had been taken from the central portion of the culture where it was dense, growth was extensive. The tissue used in these experiments was derived from a 4 month old strain of fibro-

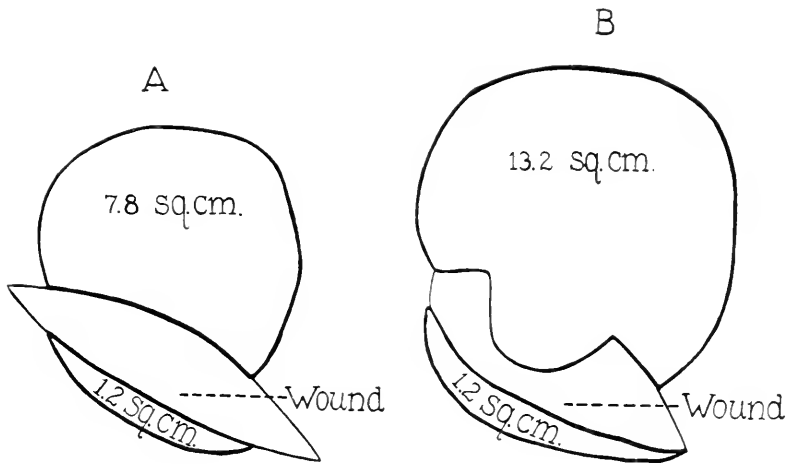


Fig. 32.

A, outline drawing of the culture shown in Fig. 33 after incision. The limits of the mother culture, the separated culture, and the wound are to be seen. B, outline drawing of the same culture after 24 hours incubation. The mother culture has almost doubled in size since the wound was made but the separated fragment has ceased to grow.

From The Journ. of Exp. Med. 1923, XXXVIII, 671.

blasts, isolated and cultivated in the *Institute of General Pathology, Copenhagen*. The small fragments were obtained by clean cuts with a sharp cataract knife. After a little practice fragments could be cut so small that they could only just be distinguished with the naked eye.

Here it might be objected that the poor growth of the fragment containing only scattered cells was a result of the

retraction of the fibrin clot. In attempts to maintain uniform conditions during the separation of the small fragment from the mother culture, another technique was devised.

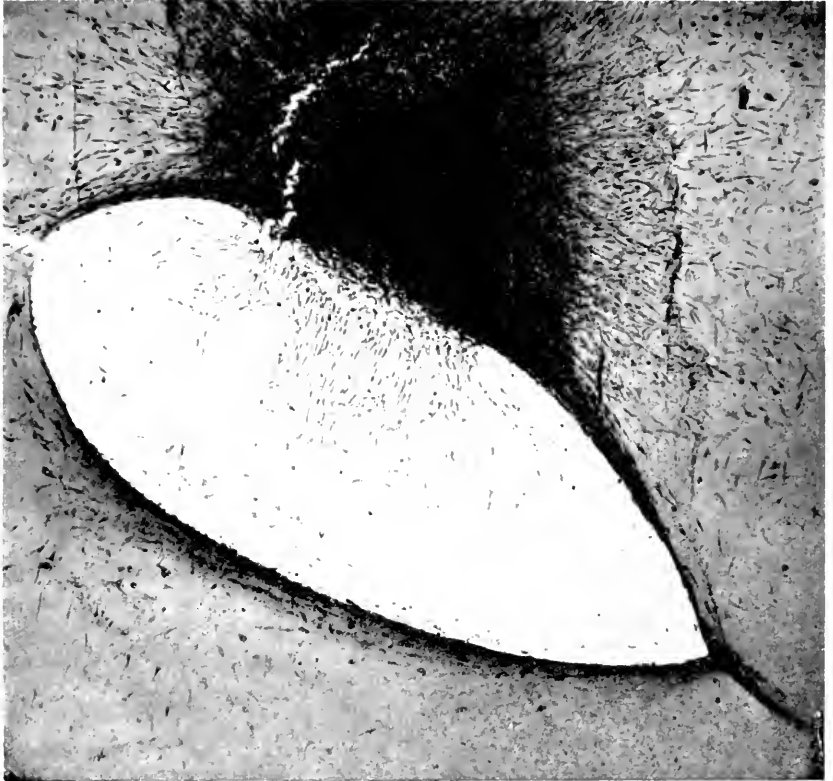


Fig. 33.

A photograph of the culture illustrated diagrammatically in Fig. 32.
From *The Journ. of Exp. Med.* 1923, XXXVIII, 671.

A culture was permitted to grow for about 20 hours, and then the cover-glass was unsealed and an incision was made in the periphery of the new growth with an ordinary sharp cataract knife, in order to separate from the mother culture a piece of tissue at the periphery where the cells were rather

scattered. The cover-glass was replaced on the slide, it was sealed with paraffin, and an outline drawing was made of the tissue — of the big piece as well of as the small one. The culture was returned to the incubator for another 30 hours; at the end of the second incubation a new drawing was made and the area measured. It was observed that the mother culture had greatly increased in size, but the fragment, separated from it by means of the wound in the clot, consisted of scattered cells and had rather decreased in size. (Fig. 32, 33.) It was observed that a peninsula had grown on the surface of the cover-glass from the central part of the main tissue, but that the separated small piece had not increased in size, much less encroached on the open space.

The result of these experiments lead to the opinion that it is not possible for one single isolated cell, or for a few isolated cells, to multiply in culture under the conditions of experiment. The cells will live, but will not divide. We are apparently not dealing with the growth of independent cells in the tissue culture, but with a partial organism, a piece of tissue, obeying the laws of regeneration. The cells seem to be closely related, but their condition and capacity for growth seem to depend on their neighbouring cells.

In the discussion of these questions may there not be other explanations why multiplication of isolated cells does not take place in our experiments on the individualisation of tissue cells? In other words is the method for the isolation of living tissue cells, above indicated, so favourable that we can assure ourselves that the cells are in good condition after transference?

Some features which perhaps may play a more or less important part should now be mentioned. It has been shown that in transferring tissues from one culture to another it is important to put the tissues in the medium so that the cells are in close contact with the medium. If we cut into a piece of tissue in such a way that a bit of coagulated plasma from

the old culture medium is interposed between a part of the new medium, in most cases no growth is produced in that part. When a piece of plasma is left attached to the culture, growth takes place at the edges first, where the cells are directly in contact with the new medium. This may be due to two factors: firstly, the old piece of plasma may contain so many decomposing products left over from the first growth, that it produces unfavourable conditions from the very beginning; and secondly, a purely mechanical effect is produced, namely a shrinkage or retraction of the plasma clot which before adhered to the cover-glass, and which after it has been cut shrinks on account of its elasticity. This shrinkage changes the structure and the relation between the fibrin and fluid and between the cells and fibrin meshwork. These facts may explain why no growth is present under the condition of experiment. — By making an incision in the expanded plasma coagulum, possibly the fibrillary structure is changed. Retraction may take place after the incision is made. It was observed that the incision created a large elliptical wound, very similar in appearance to certain incisions made in the skin.

We do not know the final structure of the fibrin meshwork and we do not know how the system of the plasma clot is built up with regard to the solid and liquid phases. It might be possible, however, that the absence of growth is due to the mechanical changes.

To return now to the experiments with isolated cells, it is possible that the vitality of the cells suffers during manipulation; but all signs seem to indicate perfect vitality, since a lively ameboid movement is present and a quick transformation from a round cell-shape to a typical spindle shaped fibroblast occurs. There is, however, a possibility, that a single cell may suffer from incarceration in the dense coagulum; and it may be unable perhaps to produce a sufficient liquefaction of the medium in its immediate surroundings.

It is essential to discuss all possible influences, which may cause technical errors. There seems anyway to be certain laws which bind the cells together.

In all well-stained preparations of well-grown cultures of tissue cells, the cells are found to be connected with each other by protoplasmic adherences Fig. 34 and 35. Under



Fig. 34.

low magnification, an apparently isolated fibroblast is often found at a great distance from the mother-cells, but upon observing it with high magnification, protoplasmic processes may be observed directly connecting with other cells. Mitochondria are often observed in these connections, Lewis³³⁹⁾ and when a gold impregnation is made use of, beautiful intercellular connections are seen.

The intercellular fibrils mentioned above, which are found in the tissue cultures, are perhaps not the same as the connective tissue fibrils found in the living organism. The latter seem to have their origin in a purely mechanical process, which Baitzell ⁷ was able to produce in a plasma clot without the presence of living tissue. On the contrary, the intercellular fibrils, which are found between the single cells in the culture, play perhaps a vital rôle in



Fig. 35.

relation to the condition of the cell itself. They are not usually found in dying cultures. An indication of the approaching death of a tissue is the emigration of unusually large-sized spherical cells. These cells when closely observed under high magnification showed no protoplasmatic connector between them and even with the greatest care, recovery is very difficult to bring about after this stage has been reached. There is no doubt whatever, that the protoplasmic bridges which exist between the tissue cells *in vivo* the *plasmodesma*, play a vital rôle, and they

are found to be very strong in tissues which have a vital significance.

The embryonic tissue juices contain substances which promote growth in vitro for an indefinite length of time ⁴⁶¹. Extracts of tissues from the adult organism also possess growth-promoting substances but not of the same grade as the embryonic tissue juices. Carrel ⁵⁸ has shown that certain tissues give an extract with greater activating power than others. Extracts of leucocytes or spleen possess nearly as much activating substance as embryonic tissue juice ⁵⁸. The mechanism of growth and multiplication of tissue cells is a profound and complex phenomenon. Besides the growth-promoting factors found in the tissue juices, there may possibly be something, produced in the body of the living cell or certain cells only, which initiates cell division and is transported directly from one living cell to another. Haberlandt ^{211 242} found that certain cells in the plant tissue, the leptom-cells, liberate substances, which initiate the cell division. Also Akamatsu ¹ showed that substances stimulating the cell division occurred in the blood, when a wound is made.

The cytotropic character of tissue cells has already been noted by several investigators. Roux ⁴⁵⁸ was the first to describe it. Recently Roux ⁴⁵⁷ has noticed the striking ability of isolated tissue cells to reunite. He never saw any division or multiplication of isolated single cells. Also Burrows ⁴¹ mentions the important relation of cells in tissue cultures to each other. He expresses himself as follows: "The tissue culture cannot be compared in detail with the bacterial culture. The tissue cells planted in plasma do not grow at the expense of the plasma. Single cells may show movement in this medium, but they do not grow Growth takes place only about fragments of tissue The nutrient material for the growth of these cells comes from the cells disintegrating within the fragment." That the nutrient material for the growth of tissue cells comes from

the cells disintegrating within the fragment, Burrows^{36, 37} & ³⁸ has recently shown. By establishing an active current in the hanging drop culture of fixed tissue cells, by washing continuously the plasma clot with a stream of serum, these stimulating substances from the disintegrating cells are washed away, the degeneration of the central portion of the fragment is delayed—as also is the migration and multiplication of the cells. On the contrary lymphoid cells from the spleen under the same conditions migrate entirely out as lymphoid cells and the whole fragment of spleen disappears. In the simple hanging drop culture, such a complete dissipation of the cells never occurs from the spleen fragments. The migration of these cells is increased by the flowing serum. The stimulating substances according to Burrows are properly speaking the same as the growth-promoting substances in the embryonic tissue juice.

Besides these stimulating substances found in the embryonic juice, in disintegrating tissue cells, things seem to indicate, that there is something in the mass of tissue, which controls the growth and proliferation of the cell individuals. It is observed here, that the activating substances in the embryonic tissue juice are not the only factor necessary for the growth and multiplication of the isolated tissue cell. This stimulus which initiates the cell division, is probably not of environmental nature, but is produced within the cytoplasm of the cells and transported within the cytoplasm directly from cell to cell. If this is true, and if the proliferation of the cell is controlled by the bulk of tissue, we would expect to find a kind of rhythmic setting in of cell divisions in a piece of growing tissue in vitro.

What I am trying to discover in this study of tissue culture, is whether we are dealing with more or less independant cells or with a piece of tissue obeying the laws of regeneration, i. e. colonies of cells, in direct dependance upon one another, as is the case in organisms.

A favourable method for isolating tissue cells and later embedding them in a suitable fixed medium, has been worked out, and still it has not been possible to observe cell division when the cells were isolated. Multiplication took place only in those cases where several individuals were in close contact with each other.

A further study has also been undertaken to determine the vitality and ability to proliferation of the different parts of the tissue cultures. It was ascertained that when these parts contain few or scattered cells, no growth took place, but when the parts contained cells in close contact with one another, growth took place after the parts had been transferred into a new medium. The healthy condition and the signs of permanent life in these isolated cells, seem to point to the urgent necessity for direct contact in producing multiplication. However, other explanations of this phenomenon have been discussed. The direct protoplasmic connections, which are always found in the well-grown cultures, and the cytotropic character of the tissue cells, speaks directly in favour of the assumption, that the cells are individually dependant on one another.

In order to go deeper into the problem and endeavour to prove the suggestion, that divisions of cells take place only when several tissue cells are present and in good protoplasmic contact, it was important to know a little about the number and percentage of cells undergoing divisions at a given moment *in vitro*, and to locate these dividing cells in relation to cells which have divided previously.

To attack the problem different methods were devised. Some preliminary experiments were undertaken. Several times I have been struck by the fact, that some specimen of cultures contained many mitotic figures and at other times specimens were found to contain very few, or at least it was difficult to find any. I have noticed great difference in the numbers of mitosis in cultures of fibroblasts as well as in cultures of epithelium and cartilage.

The cultures used in all the experiments were derived from a 9 months old strain of chicken fibroblasts. The strain was isolated and cultivated in the Institute of General Pathology of the University of Copenhagen.

The preliminary experiments were briefly made in the following way. Single, good cultures of fibroblasts were cut into 1-6 pieces of equal size and each of the pieces kept as individual cultures under identical conditions for 24 hours. With various intervals from half an hour to four hours, two cultures, derived from the same culture, were fixed and stained simultaneously. Two cultures were fixed simultaneously to let them control one another. — In these stained specimen, the cells in any stages of division were counted in the line of periphery, and after that all the tissue cells in the line of periphery and the percentage of cells in a stage of division were figured out. The line of periphery of the growing piece of tissue is a rather definite thing. As peripheral cells only those were considered which were outermost, i. e. cells with the one end in contact with another cell and the other end free.

The few experiments of this kind gave only a weak confirmation of the earlier observations which should indicate that the percentage of dividing cells differ from time to time. The two cultures taken out and fixed simultaneously proved to have a percentage of dividing cells very much alike within the accuracy of the experiments.

The result of one of these preliminary experiments can be seen in the table 12. The average percentage of mitotic cell divisions going on at a given moment under the experimental conditions was found to be about 2 per cent. The figures for the percentage of the two or three cultures fixed at the same moment are not so absolutely bad. The experiments gave only a rather weak indication that there, at a certain moment, are many cells dividing and at other times relatively few.

In order to get a better technique and more accurate information as to the function of such rhythmical cell division, another method was applied. Series of photomicrographs were taken of parts of the peripheral line of growing fibroblasts in the culture.

Table 12.
Experiment No. 460 and 461.

No	Total number of mitosis in periphery.	Total number of cells in periphery.	Percent of mitosis.
460—1	8	240	3,3
460—2	8	322	2,5
460—3	6	283	2,1
460—4	9	380	2,3
460—5	14	386	3,6
460—6	9	338	2,6
Average:			2,7
461—2	3	157	1,9
461—3	2	268	0,8
461—4	4	344	1,2
Average:			1,3

This table shows the total number of mitotic figures found in cultures of fibroblasts, fixed and stained. The mitotic figures among the cells representing the total line of periphery of the culture can be seen. Cultures Nos. 460—1—2—3—4—5 and 6 are made from one culture. The cultures Nos. 461—2—3—4 are also made from one culture.

Nos. 460—1 and 460—2 were taken out from the incubator at the same time and fixed immediately. Half an hour later Nos. 460—3 and 460—4 were taken out and fixed. In another half hour Nos. 460—5 and 460—6 were taken out and fixed. An hour later Nos. 461—2, 461—3 and 461—4 were fixed.

The technique was simply this. A culture of fibroblasts was incubated for about 24 hours and thereafter placed under the microscope, kept at a temperature of 39° C. The size of the photographs was 4.5 by 6 cm. and were taken

Table 13.
Experiment No. 688.

Plate No.	Time	Number of cells in division in periphery.	Number of cells in periphery.	Percentage of cells in division.
1	10,32	2	26	7,7
2	10,42	Spoiled.....		
3	10,52	1	27	3,7
4	11,02	0	25	0,0
5	11,12	0	25	0,0
6	11,22	1	28	3,6
7	11,32	1	31	3,2
8	11,42	1	32	3,1
9	11,52	0	35	0,0
10	12,02	1	35	2,9
11	12,12	3	35	8,6
12	12,22	0	35	0,0
13	12,47	1	34	2,9
14	12,57	0	35	0,0
Average :				2,5

Table 14.
Experiment No. Journ. 109.

Plate No.	Time	Number of cells in division in periphery.	Number of cells in periphery.	Percentage of cells in division.
1	1,50	0	38	0,0
2	2,00	0	41	0,0
3	2,10	0	42	0,0
4	2,20	0	39	0,0
5	2,30	0	39	0,0
6	2,40	Spoiled.....		
7	2,50	0	39	0,0
8	3,00	0	39	0,0
9	3,10	0	40	0,0
10	3,20	1	38	2,6
11	3,30	0	40	0,0
12	3,40	3	38	7,8
13	3,50	0	39	0,0
14	4,00	0	37	0,0
15	4,10	1	38	2,6
16	4,20	0	40	0,0
17	4,30	0	40	0,0
18	4,40	1	40	2,5
19	4,50	0	40	0,0
20	5,00	0	41	0,0
Average :			39	Average : 0,8

Table 15.

Experiment No. Journ. 110 a (Culture No. 1820 2).

Plate No.	Time	Number of cells in division in periphery.	Total number of cells in periphery.	Percentage of cells in division.
1	1,30	0	35	0,0
2	1,40	Spoiled.....		
3	1,50	0	36	0,0
4	2,00	0	34	0,0
5	2,10	0	33	0,0
6	2,20	0	34	0,0
7	2,30	0	32	0,0
8	2,40	3	34	8,8
9	2,50	0	32	0,0
10	3,00	0	34	0,0
11	3,10	0	32	0,0
12	3,20	0	33	0,0
13	3,30	0	32	0,0
14	3,40	1	35	2,8
15	3,50	0	33	0,0
16	4,00	0	33	0,0
17	4,10	0	33	0,0
18	4,20	0	33	0,0
Average:			33	0,7

with the Zeiss phoku apparatus. Every ten minutes an exposure was made. Hauff's "Flavin plate" was used. The time of exposure was about 20—30 seconds.

It was observed several times before, that the process of mitotic cell division lasted 12 minutes in the line of periphery at a temperature of 39° C., therefore the ten minutes interval between exposures was employed, in order to get all the cells in the division on the plates. After having made the negatives, the examination of the material was commenced. On the negatives or prints the peripheral cells were systematically examined, i. e. every single peripheral cell was carefully studied and followed from negative

Table 16.
Experiment No. Journ. 111 b.

Plate No.	Time	Number of cells in division in periphery.	Number on cells in periphery.	Percentage of cells in division.
1	1,00	1	34	2,9
2	1,10	0	36	0,0
3	1,20	0	34	0,0
4	1,30	0	36	0,0
5	1,40	0	37	0,0
6	1,50	0	39	0,0
7	2,00	0	39	0,0
8	2,10	0	35	0,0
9	2,20	0	38	0,0
10	2,30	1	36	2,7
11	2,40	1	37	2,7
12	2,50	1	34	2,9
13	3,00	0	33	0,0
14	3,10	2	36	5,5
15	3,20	1	33	3,0
16	3,30	0	32	0,0
17	3,40	0	31	0,0
18	3,50	0	35	0,0
19	4,00	1	33	3,0
20	4,10	0	36	0,0
Average:			35,2	1,1

to negative throughout the entire series of exposures, to see if it should divide somewhere. The cell divisions could always be controlled by the negatives in succession. The dividing cells were always checked at the earliest stage so as to get the time for the division for the comparison. After a little practice it was so easy to distinguish dividing cells, that it could almost be foreseen when a cell was going to divide at the very next moment.

This method gave a much better and truer picture of the phenomenon. The only disadvantage was, that we were not dealing with as many cells in the microscopical field

Table 17.

Experiment No. Jour. 112 a (Culture No. 1928 = 2).

Plate No.	Time	Number of cells in division in periphery.	Total number of cells in periphery.	Percentage of cells in division.
1	11,00	2	39	5,1
2	11,10	1	40	2,5
3	11,20	0	42	0,0
4	11,30	0	39	0,0
5	11,40	1	41	2,4
6	11,50	0	43	0,0
7	12,00	0	40	0,0
8	12,10	0	41	0,0
9	12,20	0	39	0,0
10	12,30	0	40	0,0
11	12,40	1	42	2,3
12	12,50	0	44	0,0
13	1,00	4	44	9,0
14	1,10	2	42	4,7
15	1,20	0	41	0,0
16	1,30	0	40	0,0
17	1,40	0	42	0,0
18	1,50	0	41	0,0
19	2,00	0	42	0,0
20	2,10	0	42	0,0
21	2,20	0	44	0,0
22	2,30	0	40	0,0
23	2,40	0	42	0,0
24	2,50	0	43	0,0
25	3,00	1	42	2,3
26	3,10	2	45	4,4
27	3,20	0	41	0,0
28	3,30	1	41	2,4
29	3,40	0	42	0,0
30	3,50	0	40	0,0
31	4,00	1	42	2,3
Average:			41	1,2

Explanation to the text figs. 36, 37, 38 and 39.

In all the figures the ordinates represent the percentage of peripheral fibroblasts in state of division. The percentage is figured out from the number of peripheral cells observed on the corresponding negative. The abscissae represent in time the interval of 10 minutes.

as by the former method, in which we were able to oversee the total line of periphery; the two methods control one another in a very satisfactory manner.

The results of these experiments, as it can be seen on tables 13, 14, 15, 16 and 17 and the diagrams Figs. 36, 37, 38 and 39 are, that periods of high percentage of cells in

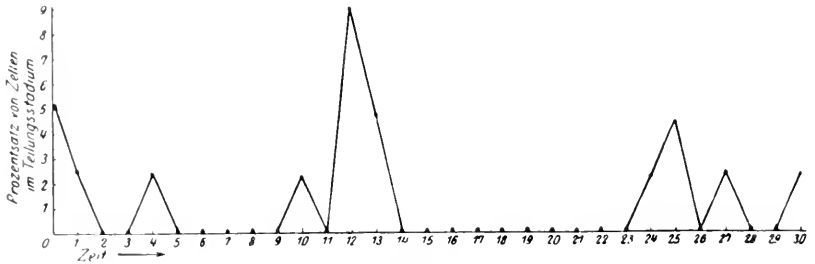


Fig. 36.

stages of division are followed by periods of low percentage or none. The figures obtained by this method can of course not be taken too literally on account of the rather rough method, but it is interesting to follow the great changes

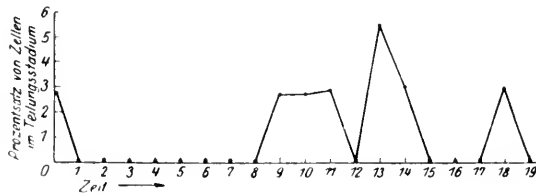


Fig. 37.

from time to time. It was always observed, that long periods with few or no dividing cells are followed by periods with many dividing cells. In the fig. 36 it can be seen very strikingly, that before and after a big rise in the percentage of dividing cells, there is a rather long period of latency.

The results of these experiments speak also very much in favour of the assumption that rhythmic impulses of some kind are passing through the regenerating piece of tissue in vitro and that there exists an interdependency of the

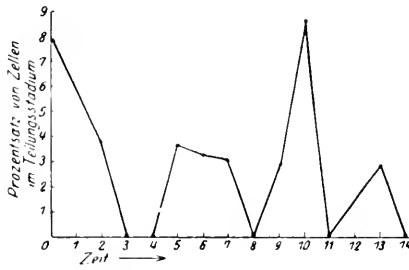


Fig. 38.

cell individuals on each other. In other words the explanted tissue seems to obey the laws of the physiological integrity.

If the entire material from the series of exposures is collected, the total number of cases examined are 100 Table

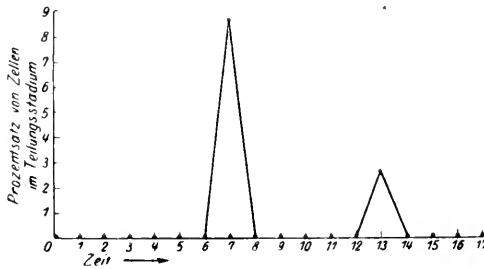


Fig. 39.

18). The photographs show an average of 37 peripheral cells in the field. In table 18 the frequency of mitotic cell divisions on an average of 37 cells can be seen. The result of these figures is, that an average of 2 % of the cells are in mitotic division at a given moment.

Table 18.

Number of mitotic figures in the periphery of an average of 37 cells.	Frequency in per ct.
0	64
1	19
2	6
3	6
4	4
5	0
6	1
Total: 100	

This corresponds very well with the results obtained by counting the mitotic figures in the total line of periphery in the stained cultures by the first method.

If T is the average time between two succeeding divisions of the same cell; and N is the total number of cells observed, we get the following expression for the number of cells $\frac{dN}{dt}$ which are dividing in a unit of time:

$$\frac{dN}{dt} = \frac{1}{T} N$$

If the time-unit, for the sake of simplicity, is set equal to 12 minutes, i. e. equal to the time the process of mitotic division on an average takes, the expression can be changed to:

$$\frac{n}{N} = \frac{1}{T}$$

where n is the number of mitosis in a given moment. As mentioned before $\frac{n}{N}$ is equal to 2%; the equation will then be:

$$T = \frac{100}{2} \cdot 12 \text{ minutes} = 10 \text{ hours.}$$

There is on an average, 10 hours between two succeeding divisions of the same cell; in other words, under the experimental conditions and under the conditions of growth in the periphery of a culture, a given number of cells will be doubled after 10 hours. If the cells were growing in a single layer, consequently the area of the culture would have increased to its double size after 10 hours. Approximately, it is the same order of magnitude which is obtained by the planimetric measurements of cultures by Carrel and Ebeling.

These experiments have brought out one main fact, namely, that the cell divisions in a given piece of growing tissue *in vitro*, an old strain of fibroblasts, seem to proceed periodically and exhibit a form of rhythm. It was observed, under the conditions of experiment, in a regularly growing piece of tissue *in vitro*, that a high percentage of cells divide within a comparatively short time and that there, as well before as also after, is relatively a long period of latency.

It is conceivable to explain the phenomenon as the result of a controlled action from several of the neighbouring cells with which the cells, about to divide, are in direct contact. The rhythmic setting in of a large number of cell divisions should according to this denote that the same impulse or principle induces numerous cell divisions in the explanted tissue simultaneously.

The phenomenon cannot be explained as the result of environmental conditions, because it was found, that single cells, which possessed all the very best conditions for life and multiplication, plasma and embryonic tissue juice, were never found to divide unless in good contact with several other cells. These experiments seem furthermore to indicate, that the growing tissue cells *in vitro*, are under the control of the neighbouring cells; we are, in other words, dealing with a partial organism and not of independant cell individuals.

It is well known, that the number of mitosis going on within the organism in certain organs or tissues, at certain periods of development, is constant. It would therefore be more unusual and unexpected if the tissue cells behaved differently in vitro. Gurwitsch^{237) 238)} found an absolute synchronicity for the division of cells in organism, in certain synzytia.

For plant tissue G. Haberlandt^{241) 242)} has observed similar phenomenons as we have described here. By cultivation of plant tissue cells in vitro, he observed, that single, isolated plant cells were not able to divide, and they showed an absolute tendency for reunion in vitro. These cells were found to divide only, when they were in contact with, and under the influence of certain other plant tissue elements, the leptom cells. In the opinion of Haberlandt, the cell division of plant tissue cells should depend upon the so-called "wound-hormons" liberated from the leptom cells in combination with the lesion. Perhaps the leptom cells play the same rôle for the plants as do the leucocytes for animal organisms. Carrel⁶⁸⁾ found, that the leucocytic secretions contained very active growth-promoting substances. Recently Akamatsu¹⁾ showed, that after having made a wound, the plasma from the wounded animal possesses considerably more growth-promoting substances than before the wound was made.

The preliminary experiments undertaken here, attempting to show that there exists a kind of periodical setting in of cell divisions in the tissue in vitro, must of course be taken with all possible reservation. It would be rather queer if this should not be the case in the explanted tissue as it seems to be proved with regard to the tissue in vivo. Regaud, Stalfeldt¹⁸⁷⁾, Gurwitz²³⁷⁾. — The figures in the experiments must not be taken too literally; the errors are of course rather gross. It has been extremely difficult to find a suitable technique for the elucidation of this problem. At the present time I have determined to

publish the results obtained so far, because it is anyhow possible to conclude, I believe, that such a rhythmic process of cell divisions is going on in the explanted tissue too. In lack of better methods at the present time, I feel that the experiments have some value. As soon as I am able to work out a better technique, the experiments will again be taken up. Micro-cinematographs will probably be the technique for this investigation.

A series of experiments have been undertaken in order to demonstrate the important rôle played by the protoplasmic bridges connecting the individual tissue cells in vivo as well as in vitro. For this purpose I have devised a new technique to discover if these protoplasmic bridges between the cells are the itineraries for impulses or stimuli, other than those described above, for the cell multiplication. My intention was to devise an experiment which easily could be repeated by other investigators and clearly would demonstrate the significance of the protoplasmic connections between the cells. Quite lately it was claimed by Charles F. de Garis ²²⁰⁾ that the cellular connections in vivo as well as in vitro were only apparent. He never observed any true connection between the cells; he never found any transport of cytoplasmic granules or vacuoles from one cell to another. This is of no importance. It is not necessary that the exchange of substances should be visible for microscopic observation. It is a general fact, anyhow, that the contact between various nerve fibres plays a rôle for the conduction of nervous currents.

It was therefore attempted to obtain a synchronous or simultaneous contraction of two separate fragments of heart in vitro, to demonstrate whether a cellular contact between the fragments could be reestablished. There was every reason to believe, that if anastomosis between the contractile elements of two pieces of heart could be established, a simultaneous contraction of the two fragments would result.

The fragments of heart were obtained from chicken embryos about 8 to 10 days old. In some experiments the fragments belonged to different embryos and in some they were derived from the same heart, whilst in other experiments two fragments derived from the same heart and their cut edges brought into close proximity. This was done in the following way. A relatively large fragment of heart was placed in the culture medium, and while the culture medium was in the liquid state, the fragment was divided by a clean cut and kept together by means of a needle and the point of the cataract knife until coagulation of the culture medium took place. Usually three fragments of heart were placed in one culture, two adjacent each other and one separate which served as a control.

Soon after the cultures were prepared, the contractions of the respective heart fragments were checked. For each of the three fragments, the intervals of time between two contractions were measured by means of a stop-watch. After 12 hours incubation the cultures were again examined and the contractions checked, 12 hours later the same thing was repeated and so on.

When several fragments of heart tissue were embedded in the same culture medium, not two of them pulsed in the same way. The individual fragments were able to pulsate exactly in the same way for long periods of time, when everything else was constant. In the experiments where the distance between the two adjacent fragments was about one mm., the zone between the fragments were filled up with the outgrowth of fibroblasts from the pieces. In a few experiments of this kind, it was observed that the individual contraction of the two adjacent fragments was not regular any longer, after the fibroblasts had intermingled in the zone between the fragments. (table 19) Before, the contractions were very regular, i. e. the intermission was the same for the same fragment for a long period of time, but after the outgrowth of fibroblasts had united, the contractions became

arrhythmic table 19. Long intermissions were followed by periods with fibrillary contractions. In the meantime the isolated, single fragment of heart had pulsed with a rather constant intermission all the time. The cultures were unsealed quickly and by means of a sharp cataract knife, the two fragments were separated in the zone of new growth. The culture was sealed again and observed under the microscope. The whole procedure took no more than one minute. From this time on, the two fragments pulsed regularly, i. e. the intermission were constant for the same fragment, table 19.

Table 19.
Culture 2013.

Time between two contractions of control.	Time between two contractions of two adjacent fragments.	Time between two contractions of the other.
Before the two became connected.		
	sec.	sec.
	2.0	1.6
	2.0	1.6
	2.0	1.6
	2.0	1.4
	2.0	1.6
After the connection.		
sec.		
3.0 3.0	1.5 2.0	1.2 1.0
3.0 3.0	2.0 2.5	20.0 0.8
3.0 3.0	2.0 2.0	1.0 0.6
3.0 3.0	2.0 1.8	0.8 0.8
3.4 3.0	2.1 1.5	1.0 0.8
3.0 3.0	1.5 2.0	35.0 1.0
3.0 3.0	1.5 2.0	1.0 0.6
3.0 3.0	1.5 1.5	0.8 0.4
3.0 3.4	1.5 1.5	0.6 0.6
3.0 3.0	1.5 1.8	1.0 0.8
3.0 3.0	1.8 1.8	0.8 0.4
	1.5 1.5	18.0 0.4
	1.8	1.0 1.0

Time between two contractions of control.	Time between two contractions of two adjacent fragments.		Time between two contractions of the other.	
	After separation.			
	5.0	5.0	8.0	8.0
	5.0	5.0	8.0	8.0
	5.0	5.0	8.0	8.0
	5.0	5.0	8.0	7.0
	5.0	5.0	7.0	7.0
	5.0	5.0	7.0	7.0
	5.0	5.0	7.0	7.0
	5.0	5.0	7.0	7.0
	5.0	5.0	7.0	7.0
	5.0	5.0	8.0	7.0
	5.0	5.0	8.0	8.0
Greatest difference before separation, 1.0			34.6	
Average difference before separation, 0.2			5.9	
Greatest difference after separation, 1.0			1.0	
Average difference after separation, 0.0			0.1	

The interpretation of this phenomenon is rather difficult. The irregularity of the contraction after the new growth of the two fragments had intermingled could possibly be explained as a mechanical effect.

In other experiments, the distances between the two fragments were not so great. The result was, that only after 18–72 hours incubation, the two fragmina pulsated as one. The space between the two fragments could after 18 hours incubation scarcely be seen and a perfect simultaneous pulsation was obtained. A type of such an experiment can be seen in table 20. In these experiments where the distances between the two adjacent fragments are rather great, it takes a relatively long time before the respective pulsation becomes synchronous. This experiment represents also the group, where synchronic contraction was obtained with two fragments of heart derived from two different chicken embryos.

Table 20.
Experiment 2040.

Time	Time between two contractions of one of two adjacent fragments.	Time between two contractions of the other.
hrs.	sec.	sec.
0	1.6	2.4
0	1.6	2.4
0	1.6	2.0
0	1.6	2.0
0	1.8	2.0
0	1.4	2.0
0	1.4	2.0
0	1.4	2.0
24	1.2	3.4
24	1.2	3.6
24	1.4	3.8
24	1.8	4.0
24	3.0	2.4
24	2.2	2.2
24	4.6	2.0
24	4.2	1.6
Interval between the synchronous contractions.		
48		
48	3.0	
48	3.2	
48	3.0	
48	3.4	
48	3.6	
48	4.2	
48	2.6	
48	3.0	
48	3.0	

A rather high percentage of success in obtaining synchronous pulsation of two fragments of heart, was observed, when the cut edges of the two respective fragments were brought close to each other and still more so, when the cut edges belonged to one another, one fragment of heart being thus

cut in two in the already prepared culture medium and kept in position there until coagulation took place. By this method a simultaneous pulsation was already obtained 21 hours after.

A good many experiments were made, to see if it was possible to obtain a simultaneous contraction between two fragments of heart, the one derived from a duck embryo and the other from a chicken embryo. The heterologous



Fig. 40.

Experiment 2066. A section through the line of contact of two fragments of chick embryo heart which had pulsated perfectly simultaneously in vitro. The contractile elements of the one are in direct contact with those of the other. x 175.

From The Journ. of Exp. Med., 1924, XXXIX, 577.

culture medium for the duck heart, namely chicken plasma and embryonic chicken juice, had no influence on the growth of the duck fibroblasts. It has been possible to cultivate duck fibroblasts for as long a period in chicken medium as 3 months. The growth of the fibroblasts from duck was just as intensive as that of chicken fibroblasts — and it is possible to say now, that the duck fibroblasts are able to grow indefinitely in the chicken plasma and embryonic tissue juice from chickens. Therefore it was of great importance

to see if such tissues, belonging to different species and both able to grow and multiply in the same culture medium, were capable of uniting in a physiological and histological integrity. It was also observed, that fragments of duck heart, were able to pulsate through several passages in the chicken culture medium.

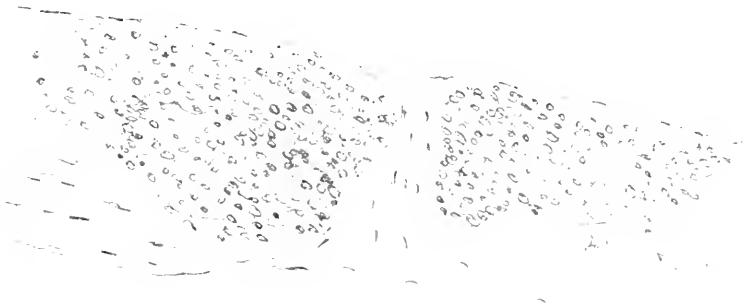


Fig. 41.

Experiment 2067. A section through the line of contact of two fragments of chick embryo heart which failed to pulsate simultaneously in vitro. A layer of connective tissue can be seen separating the contractile elements.
x 175.

From the Journ. of Exp. Med., 1924, XXXIX, 577.

The result of this experiment was, that it proved impossible to obtain any physiological union of the two different fragments.

Serial sections were made of the different cultures for histological examination. A definite histological difference could be observed in the zone between fragments, which had united perfectly and pulsated synchronously as one piece, and those which had not, Fig. 41. In the line of contact of the fragments, which united in a physiological way, the

contractile elements from the one fragment had established a direct protoplasmic contact with the contractile elements of the other fragment, whereas in the line of contact between two fragments which had not united physiologically, a definite layer of connective tissue was interposed. Fig. 11.

Two fragments of pulsating heart from the same species, cultivated in vitro, are able to unite and pulsate simultaneously. This is possible for fragments of heart, derived from two different individuals belonging to the same species, whereas it was not possible to obtain any physiological union between a heart fragment from duck embryo and a heart, fragment of a chicken embryo, though both fragments are able to pulsate and live unaffected by the culture medium. It was found also that fibroblasts from duck were able to grow and multiply indefinitely in the heterologous culture medium.

It is an interesting phenomenon, that it is impossible for two fragments of heart, belonging to different species, to unite and establish a physiological integrity, though the cell elements of the one species are able to live and multiply indefinitely in the heterologous medium. The culture medium itself does not seem to play any rôle, but the difference and the cause to the unestablished union lies in the cells themselves. The cellular contact and its function, is an important factor, differing from that played by the substances in the culture medium. If no cellular contact was obtained that could be controlled by the histological examination there was no physiological union, i. e. no simultaneous contraction.

It can therefore be concluded, that the cellular contact between the contracting elements of the two pulsating heart fragments is the unquestionable cause of the simultaneous pulsation. This is also a direct proof that the cellular contact or anastomosis plays an important rôle for this and other functions even in the tissue culture.

We may summarize the results of the experiments described in this chapter in the following way:

It is endeavoured to prove, that the tissue cells in the *in vitro* culture, are not to be compared with the bacterial culture. The tissue cells are not independent individuals, growing as such. — Each cell is an integral part of the entire mass of tissue. How small bits of tissue or how many cell individuals that are necessary for a growth and a multiplication has not yet been determined. One cell is, at any rate, not able to retain and regenerate a tissue *in vitro*.

It was also found, that a fragment of tissue, containing a few and scattered cell individuals, is not able to regenerate *in vitro*.

The anastomosis or protoplasmic connection between the tissue cells, plays a vital rôle for the continuation of the life of the cells. This is rather well known from certain tissues within the organism and now also found to be the case in the cells *in vitro*.

The experiments seem furthermore to indicate, that periodical stimulating impulses of some kind, are produced within the cells of the tissue and transported from cell to cell throughout the intercellular bridges causing the division of a large number of cells. Which cells these are, where these stimuli originate and of what nature these stimuli are, we do not yet know. It is conceivable to consider the cell division as the ultimate result of a general progressive development of a chain of tissue cells, representing various phases of the supposed stimulus or substance, which causes the cell division.

In cultures of fibroblasts, belonging to an old strain, it was found, that the cell division set in rhythmically, so that at one time a large number of cells divided, and at other times there were very few or none. This seems to indicate, that in a piece of tissue or in a portion of growing fibroblasts in a culture, several or many cells are influenced by the same principle or stimulus.

The significance of the cellular anastomosis, found in the tissue culture, is still better demonstrated in the experiments with the pulsating fragments of heart *in vitro*. It was found, that two fragments of pulsating heart tissue, either from the same heart or from different hearts of the same species, were able to reunite and the two pieces established one simultaneously pulsating unit. It was observed, that when no simultaneity of two fragments could be obtained, no histologically detectable protoplasmic connection between the contractile elements of the two fragments could be seen.

3. INTERACTIONS OF THE TISSUE CELLS *IN VITRO*.

From experiments on regeneration of certain lower animals, it is known how parts separated from the body are able to develop and form a perfect organism. It is stated that it is the separation or isolation of the part from the influence of the organism as a whole, which initiates the process of regeneration.

It has been further shown that if certain organized tissues as kidney, thyroid and skin, for instance, are explanted and allowed to grow for some length of time in cultures, the tissues loses its characteristic architecture and one type or another of the cells grow out from a tissue without any regular organization, (Chamby¹²³).

At other times the growth of the explanted complex tissue is controlled and continues to grow as such. This is demonstrated by Thomson¹⁹³ and Fischer¹⁹⁵. Thomson observed, that if a toe from a chicken embryo was explanted, an uncontrolled proliferation of cells began from the injured parts. He stated, that when the basal membranes are injured, the cells commence growing out into the medium, whereas an uninjured organ continues to grow as a complete structure, controlled by the laws of the organism. This does not seem to be a constant phenomenon; it was observed (Fischer¹⁹⁵) that small fragments of intestine from the chicken embryo did not very often exhibit any

uncontrolled growth from the beginning, in spite of the fact, that such fragments of intestine contain at least two injured ends.

Rather little is known of the interaction of the various tissues. It is of great importance to know about the laws which control the rate of proliferation and the limits of migration of the various tissues: in other words it is important to investigate the correlation of the tissues, in order to understand a little about the numerous pathological processes.

In a previous chapter, we have described a factor which plays an important rôle for the multiplication of the tissue cells. It was observed that single isolated cells are not able to divide and multiply, unless in direct protoplasmic relation to other cells of the same kind. It was also observed, that the stimulus regulating the simultaneous contraction of a heart fragment, is transported from one cell to another through the protoplasmic anastomosis of the muscle cells. It was found, that the stimuli leading to the simultaneous contraction of the fragment, were blocked by the interposition of fibroblasts.

This fact leads us to assume, that specific impulses are not transported from cells of one type to cells of another. This assumption may probably be true also as regards the growth stimulating factors, transported from cells of one kind to cells of another. In other words, a single epithelial cell may not impart to a single fibroblast the stimulus for cell division. It was found by other investigators, that protoplasmic connections between epithelial cells and fibroblasts were not seen in the organism. These phenomena are still unknown, but investigations have already commenced. If it be true, that the stimuli inducing the cell division, are specific for the various cell types, we should be able to understand a good deal more of regeneration, transplantation and other related processes.

Many unknown factors of the transplantation may be explained that way. The reason why homologous and heterolog-

ous transplants deteriorates after shorter or longer periods, may be the impossibility of establishing a physiological link of the transplanted cell-individuals with the cells of the new host, (cf. the experiment on duck and chicken heart p. 185). Many of the tumor transplants which will not take, may also be the result of a missing establishment of a physiological contact with the new organism. The athreptic theory of Ehrlich may be explained in the same way. The mouse carcinoma, when transplanted to a rat, will grow and persist for a time and will then progressively disappear. It lives and grows at the expense of the transplanted tumor cells themselves and the humors from the new host do not perhaps even inhibit the growth of the tumor cells, but the unknown, the specific stimulation for the multiplication of the tumor cells cannot be obtained in the new host. It is, of course, a more or less theoretical conclusion, which has yet to be proved.

In a former chapter of this book I have mentioned some experiments undertaken in order to settle definitely, that epithelium, cultivated for a long period of time *in vitro*, still remains epithelium and behaves as such. — The interaction of fibroblasts belonging to a ten years old strain and epithelium of a three months old strain was studied *in vitro*. Epithelium and fibroblasts were placed side by side in a culture, and allowed to grow in close proximity for several passages. — After the first 48 hours, a distinct difference was observed in the character of the two fragments. The epithelium grew as a compact mass with the individual epithelial cells in close contact. The fibroblasts migrated into the culture medium and formed a network. After a few passages the fibroblasts overgrew the epithelial fragment entirely. The combined culture showed a peripheral growth mainly composed of fibroblasts, and the only apparent indication of the presence of epithelium was that the central portion of the culture appeared semi-transparent and homogeneous and not as dense and opaque as a typical

culture of pure fibroblasts which had not been divided in the same number of generations. At this stage, the mixed cultures were divided and subcultured. This procedure was continued for seven passages and finally the preparation was fixed, sectioned and stained by Van Gieson's method. The sections showed typical epithelial and connective tissues, as found in the organism. The epithelium appeared greenish yellow in contrast with the connective tissue, which appeared pink and contained many fibrillae which were stained a decided pink. No parts of the section showed an amalgamation of the two cell types.

figs 20, 21 The epithelial cells were everywhere distinctly differentiated. In many places a definite structural arrangement of the cell elements was observed. The epithelial cells had grouped themselves to form tubules with distinct lumina. In several of the sections, the lumina could be seen filled with a homogeneous colloidal secretion. The arrangement of the epithelial cells forming the tubules resembled the conformation found in sections of salivary glands. The individual cells which formed the tubules had their nuclei disposed close to the basal membrane.

In some parts of the section, epithelial cells could be seen penetrating the surrounding layer of fibroblasts and appearing on the free surface of the tissue fragment. Fig. 21. In other parts, large masses of keratinized cells, surrounded by a layer of low epithelium, could be observed.

These experiments show that epithelium cultivated for 2 months in vitro retained its morphological characteristics which differed decidedly from those of fibroblasts. But a still more striking fact was observed; namely, that the differential stain ad modum Van Gieson brings out the chemical difference between the two cell types when they are allowed to grow together. The epithelium was observed to have formative ability; i. e., the epithelial cells arrange themselves in winding tubules. This has already been mentioned in an earlier report of experiment in which the

epithelial cells, cultivated on the free surface of the clot, grew in a single layer and were described as organizing themselves in structures which closely resembled cross sections of glands. The experiments just mentioned confirm this statement, since the tubular arrangement may be followed throughout all serial sections.

Champy¹²³ states that no strains of tissue cells can be cultivated for any length of time in vitro without a change occurring in their morphology. I have shown that fibroblasts and epithelial cells may be cultivated in pure cultures for long periods without dedifferentiation. Therefore, in this case it seems that Champy's¹²³ statement does not confirm the experimental data. On the other hand, he also states that epithelial cells in the presence of connective tissue cells do not dedifferentiate. This fact is fully substantiated by my work. The experiments in which pure strains of epithelium and fibroblasts were mixed and allowed to grow together in one culture, show clearly that normal epithelial cells are not able to amalgamate with normal fibroblasts as would be the case with cancerous epithelium. The normal epithelial cells stick together because of a certain interdependancy of the cells upon one another. If the epithelial cells were able to obtain the growth promoting principles from other cells than the epithelial, in this case the fibroblasts, the picture would probably have looked different. Chlopin¹³⁷ has always noted also that the epithelial cells were absolutely distinctly separated from the fibroblasts.

It was stated by Champy¹²³ that epithelium from embryos, cultivated in vitro, dedifferentiated within a few hours and adult tissue persisted for a long time in cultures without changing. It was also stated that the dedifferentiation of epithelial cells was delayed or did not take place at all, when fibroblasts or connective tissue cells were present.

The same statement was made quite recently by Drew¹⁵⁷. He describes how a pure culture of epithelium com-

mences suddenly to differentiate, when a portion of connective tissue is added. By differentiation the formation of rudimentary tubules is meant here. These are found to be formed in normal as well as in cancerous epithelium. — It is, in other words, the formative ability of the epithelium, which is meant by differentiation here. Drew ¹⁵⁷⁾ describes, however, the growth of epithelium as “sheets of undifferentiated tissue”. Champy ¹²³⁾ describes the dedifferentiation of the epithelial cells, going on in vitro, as a transformation of the epithelium into an indifferent embryonic tissue, not unlike fibroblasts. From this, it is not quite correct of Drew ¹⁵⁷⁾ to call the epithelium growing in sheets, undifferentiated tissue. The question of dedifferentiation according to Champy can now be considered as settled. Fischer ¹⁹²⁾ and Ebeling and Fischer ¹⁶⁷⁾ have proved that epithelium cultivated in vitro does not dedifferentiate in the sense of Champy, but can be cultivated indefinitely and behave as epithelium, morphologically and biochemically all the time.

Drew ¹⁵⁷⁾ describes also, that keratinization is a consecutive process on epithelium under the direct influence of fibroblasts in vitro.

Our experiments have shown that both phenomena described by Drew ¹⁵⁷⁾, generally take place without the presence of fibroblasts or connective tissue cells at all. Fig. 12. The strain of epithelium of our own, is a pure culture — and there had not been the slightest contamination with fibroblasts during the cultivation; nevertheless, the epithelium has “differentiated” in the sense of Drew to primitive tubules. (Fig. 15.) These kinds of “differentiations” we have observed now and then in up to 3 months old strains of epithelium which had not been in contact with fibroblasts all that time.

The same can be said about the keratinization: this is also described by Drew as a process which originates under the influence of fibroblasts.

The organisation of the epithelial cells, cultivated in vitro occurs when the growth of the culture for one or another reason suffers and becomes irregular. It may be the result, when the tissue is not properly placed in the

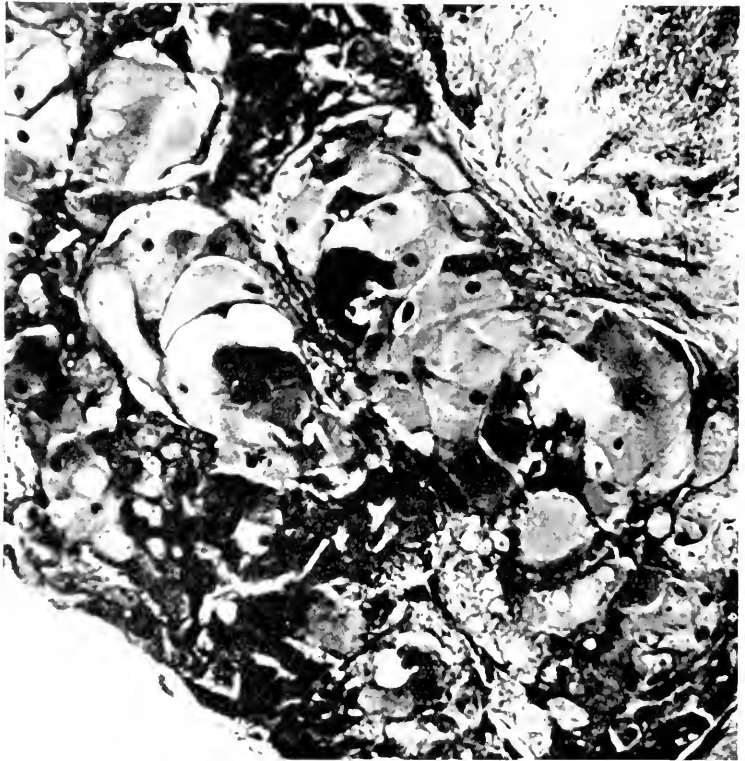


Fig. 42.

Experiment 1476. 22nd. passage of a 48 hour old pure culture of epithelium from the iris of the chicken embryos. The photograph is taken of a section through the culture and the keratinization can be seen.

From The Journ. of Exp. Med., 1924, XXXIX, 585.

medium. — or more so even when the central portion of the tissue becomes thick and more or less necrotic, because it is poorly supplied with oxygen and nourishment.

In my experience, the keratinization in epithelial cultures, is always the result of the culture suffering from lack of oxygen and nourishment. It cannot be denied, however, that the presence of fibroblasts, as Drew has described it, can give the same result, but it is only to be demonstrated here, that both phenomena occur generally without the presence of fibroblasts, and that the organisation according to Drew, the "differentiating" factor of connective tissue, is not a specific reaction of these cells on epithelium. It is nevertheless an interesting fact, that connective tissue cells, cultivated side by side with epithelium, bring about a quick organisation and keratinization of the cells; it demonstrates probably only, that the intrinsic power of fibroblasts is much greater, compared with that of the epithelium.

It is worth while mentioning here the fact that the epithelium used for the cultures is iris epithelium, which does not generally keratinize in the organism.

In order to get a suitable subject for the study of the interdependency of the various tissues *in vitro*, a series of experiments was undertaken with the cultivation of complex tissues, i. e. tissues consisting of several types of cells.

If a part of an organ or a complete embryo was explanted, it increased markedly in size and no outgrowth of cells took place. Thomson¹⁹³⁾ found that if a toe of a chicken embryo, for instance, was explanted, an uncontrolled proliferation of cells began from the injured part. When the basal membranes are injured, the cells begin to grow out into the medium, whereas an uninjured organ continues to grow as a complete structure controlled by the laws of the organism.

This statement of Thomson could not be quite confirmed by my experiments. By explanting small bits of intestines from chicken embryos I observed very seldom any uncontrolled growth; the basal membranes of the tissues in the intestine must have been injured by the excision.

My experiments of controlled growth were made on the small intestine from chicken embryos which were about to hatch, i. e. 20 to 21 days old. The purpose of the experiments was to see if it was possible to obtain portions of intestinal tissue which, by means of their epithelial lining, were able to resorb nutriment from the surroundings and supply the entire fragment with nourishment. Therefore the intestine of the old embryo was selected in order to get an epithelium which had developed the ability of breaking down foreign proteins and transporting the substances to the interior of the tissue.

TECHNIQUE.

20 to 21 days old chicken embryos were taken out of the shell in the usually way. A fragment of the small intestine was extirpated and placed in Ringer's solution. Here it was cut into very tiny fragments and placed in the culture medium consisting of equal volumes of chicken plasma and embryonic tissue juice. In some experiments the intestine was opened and a little strip was cut off and cultivated, or the fragment of intestine was turned inside out, leaving the epithelial coat outside and the serosa inside the lumen.

After 48 hours incubation the cultures were unsealed and the small fragment picked up with the point of the knife, or aspirated by means of a pipette, washed a minute or so in Ringer solution and replaced in a fresh culture medium. An extensive liquefaction was brought about after the 48 hours cultivation so the fragment was usually found floating in a cup-like excavation in the liquefied plasma

RESULTS.

It was very seldom observed, that the cells were growing out in the medium, as explants generally do manifest their actual life. After a few passages the epithelium could be seen growing all around the fragment and formed an entire coat. At this stage, the fragment had become spherical and

its surface shiny as a fresh mucous membrane. Macroscopically as well as microscopically it appeared as a semi-transparent body with a slightly denser central portion. Active peristaltic movements were observed around the edge of the body as well as on the free edge of the cuticula of the epithelial cells. A lively migration of amoeboid cells took place between the epithelial cells.

Active contraction of the intestinal muscles could be observed even after more than a months cultivation. These contractions were brought about by a slight cooling of the preparation.

The size of the bodies, or "organisms" varied according to their age. Generally, they tended to decrease in size as time went on.

The actual secretion could be seen to exude from the epithelial cells. The secretion was rather mucous, and after a few hours cultivation the body was floating around in a little lake in the plasma clot. In subsequent stages, the intestinal body became pellucid, and the coat of regular cubic epithelium could be studied thoroughly. Often it was possible to observe small appendicular cystic formations which appeared on the surface of the body within a few hours. These cysts increased markedly in size from hour to hour and it was clearly seen, that the fluid could only pass in from the exterior and not in the opposite direction. At the time when the cultures were to be transferred to a fresh medium, the cysts were injured by the operation and collapsed.

By the histological examination of the intestinal bodies after a months cultivation, they were found in an excellent state of preservation. The cylindrical epithelium had grown all around the fragment. The villi had disappeared and the surface of the body was very even and smooth. The epithelium in the Lieberkühn glands was well preserved in many cases, but did not seem to have any communication with the outside. The connective tissue cells had formed

a stroma with numerous fibrillae, (Fig. 43) There was no evidence of uncontrolled growth, as the epithelial cells did not go beyond their natural border line, and there was no intermingling of the different cells.

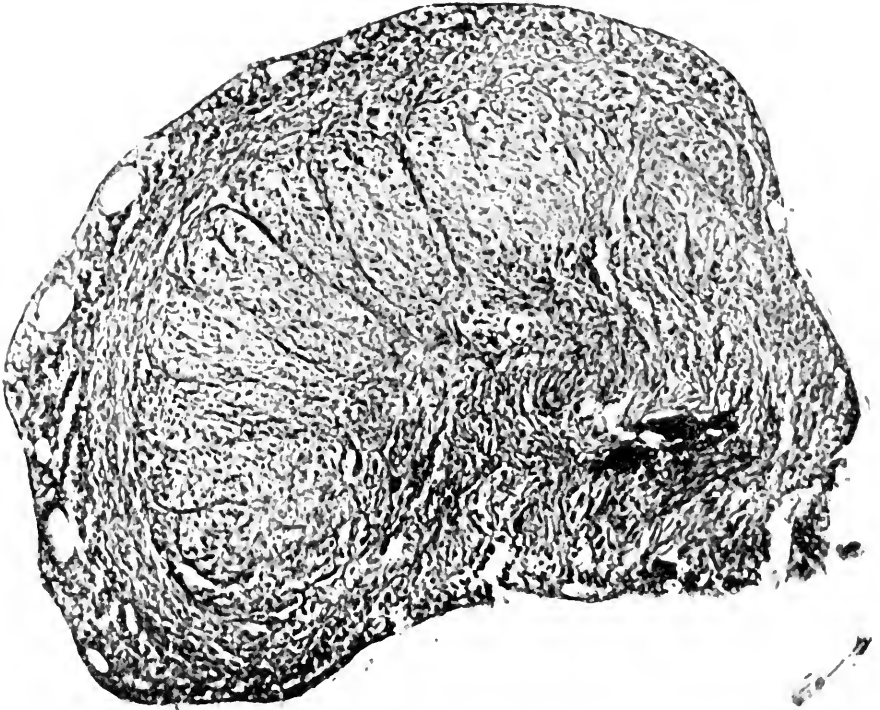


Fig. 43.

Experiment 1085—6. Section of an intestinal „Organism“ cultivated in plasma and embryonic juice for a month. x 200.

From The Journ. of Exp. Med., 1922, XXXVI, 393.

Because of the liquefaction which always occurred a few hours after the transferring to a new medium, it was supposed that such bodies were able to live in a fluid medium. It was therefore essayed to cultivate these bodies in a fluid medium consisting of embryonic tissue juice diluted

with Ringer's solution. They were not transferred to the fluid medium before the epithelium had grown all around the fragment. After a months cultivation in a fluid medium, the intestinal organisms were fixed in 2 % formol-Ringer solution and sectioned. There was a marked difference between

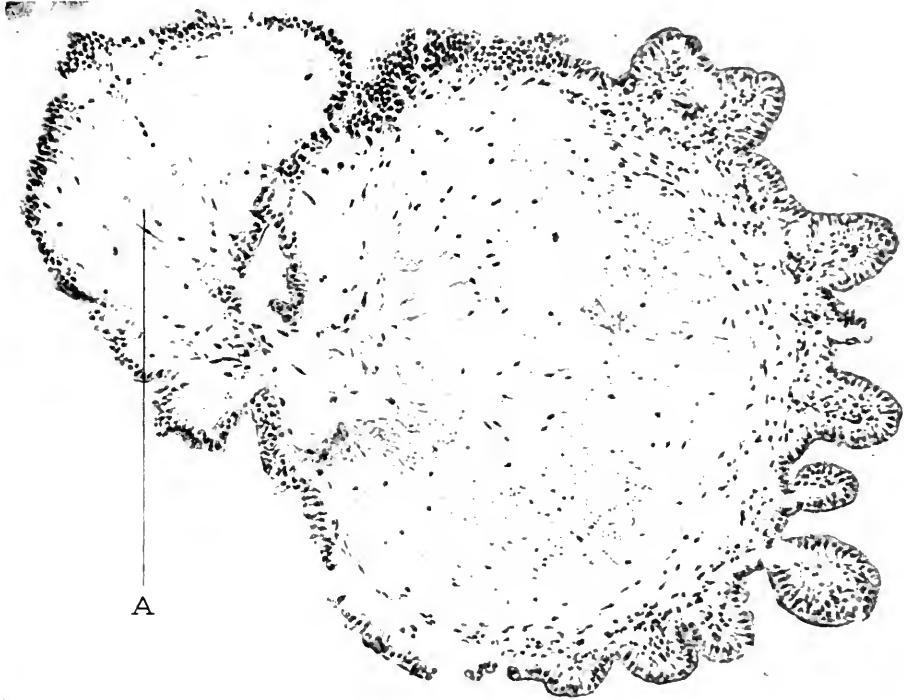


Fig. 44.

Experiment 2107 -24. Section of an intestinal „Organism“ cultivated in embryonic juice without plasma. The epithelium is normal. The stroma is very loose and the cells are sparsely represented. A cystic formation is seen at A. x 150.

From The Journ. of Exp. Med., 1922, XXXVI, 393.

those cultivated in the fluid medium and those in the solid medium. Those in the fluid medium had well preserved villi and very little stroma left. In the centre of the “organism”

only a faint trace of the stroma remained. Fig. 11. Just under the lining epithelium, which appeared to be normal, were a large number of epithelial cells in a more or less amoeboid state. Under this layer, an empty space could be seen which had probably been filled with liquid, and finally toward the centre of the body, a very thin, loose stroma with single connective tissue cells.

The cylindrical epithelium was very well preserved. Its free surface formed a continuous and solid cuticle. This contact between the epithelial cells was only perfect in their peripheral part. Between the external and the basal parts of the cells, free spaces could be seen, as if they had been formed by the pressure of a liquid within the body. Fig. 15).

The conditions under which the intestinal bodies lived in a liquid medium were probably not the very best. The correct conditions of life in a fluid medium will doubtlessly be found and perhaps it will render possible maintaining the life of these for a long time. And it will be interesting to observe whether they are able to utilize heterologous nutriment because otherwise the epithelium of the intestine is capable of breaking down complex foodstuffs.

Interactions of the different cell elements can be studied on these intestinal bodies. Transplantations have already been undertaken. Pure cultures of epithelium were grafted into such an "organism" without difficulty. The growth of malignant cells may be studied in the same way; the incipient growth of malignant cells in an organism can easily be studied this way.

Before closing this chapter, some very important results obtained by Carrel and Ebeling within very recent times, should be reported here. As the results of a long series of investigations on the physiology of the leucocytes, Carrel and Ebeling⁶⁸⁻¹⁰³⁾ have quite recently shown that the leucocytes actually secrete substances which serve as nutrition for other cells.



Fig. 45.

Experiment 2107—22. Section of an intestinal „Organism“ cultivated in embryonic tissue juice alone for a month. The loose stroma and the cubic epithelium with the dilatated spaces between the individual cells may be seen.

x 272.

From The Journ. of Exp. Med. 1922, XXXVI, 393.

The highly improved technique of cultivating tissue cells has made it possible to study several physiological problems hitherto unassailable. Carrel ⁶⁶ developed recently, as already described, a new technique by which the culture medium may be renewed frequently without any mechanical disturbance of the tissues. With this technique, epithelial cells, fibroblasts and leucocytes may be observed in a condition of uninterrupted growth for about a month. Therefore the properties of the leucocytes could now be investigated.

It was observed that the leucocytes cultivated in the special flasks mentioned already, secreted substances which was found to promote multiplication of fibroblasts and epithelium. When small amounts of foreign protein was added to the culture medium, the leucocytic secretions acquired a still greater growth-promoting power. It is shown by Carrel ⁶⁷) that leucocytes secrete growth-promoting substances as well in vitro as in vivo. Carrel has called the leucocytes *trephocytes* i. e. nurses of the fixed cells, as they secrete the *trephones*. The leucocytes are found to synthesise growth-promoting substances from certain constituents of serum - a process, which fibroblasts or epithelial cells are not able to imitate. This is demonstrated by Carrel in his beautiful experiment in keeping colonies of fibroblasts and leucocytes in large flasks in plasma only. After a few days the colony of fibroblasts appeared very degenerated and were almost dying. The colonies of leucocytes soon increased in size and reached the outline of the colony of dying fibroblasts. Immediately after the fibroblasts were reached by the leucocytes, they became rejuvenated.

An important contribution to the physiology of the tissue cells is here given by Carrel, since he has proved the old conception of Claude Bernard that the internal secretions are nutrient, secretions such as glycogen, albumin and fibrin. Claude Bernard and later Renault con-

sidered the leucocytes as cells playing a capital rôle as mobile unicellular glands, carrying the food material to the fixed cells. Jolly concluded from his investigations on the lymphoid apparatus that lymphocytes store important chemical substances, which are used by the organism.

The existence of such substances is now demonstrated by Carrel, who called them trephones, and they are defined as substance, which are used by the cells directly for the synthesis of their protoplasm. On the contrary, the hormones are defined as substances which stimulate other cells to activity. They act as catalysts, but do not build up protoplasm. Both trephones and hormones are required for the growth of tissue.

It has often been mentioned in this book that the normal tissue cells, fibroblasts and epithelial cells are not able to synthesise their protoplasm from the serum constituents. They require substances called trephones, which are present in embryonic tissue, certain glandular tissue and in the leucocytes; the latter being able to synthesise these substances from the serum compounds. Fibroblasts and epithelial cells are maintained in a true state of cultivation only when embryonic tissue juice is added to the culture medium. Lymphocytes and large mononuclear and certain malignant tissue cells behave in entirely different manner. According to Carrel, the monocytes die in a short time when cultivated in embryonic tissue juice. In diluted serum they remain alive for a much longer period of time. It is clear that they build up protoplasm from some of the compounds of serum, a thing which fibroblasts and epithelial cells are unable to do. Fibroblasts cultivated in a medium containing embryonic tissue juice as the only source of nitrogen multiply extensively. In the latter case, there is no doubt that the embryonic juices do not act as hormones, but actually take part in the synthesis of protoplasm.

The conclusion of Carrel's investigation according to Carrel himself is, that the lymphocytes both possess

the function of nourishing tissues, besides that of disintegrating foreign substances, necrotic cells, bacteria *a. s. o.*

According to their condition, leucocytes liberate substances of varied nature, trephones are secreted by normal cells, or diffuse from dead cells before they disintegrate. Trephones probably disappear at a certain stage of cell degeneration, according to Carrel, and the substances which diffuse from disintegrating leucocytes are often toxic for the tissue cells. Extract from pus generally depresses the multiplication of cells. Carrel has observed *in vivo*, that the cicatrization of wounds is delayed when an abscess in some part of the body is present. Similar observations were made when sterile pus was injected subcutaneously while the healing of the wound was observed.

According to Carrel pathological processes are often characterized by a reactivation of tissues which possibly for many years have ceased to multiply. There has always been a question as to what determines an increase of the growth energy of a tissue when it is needed. From the experiments of Carrel we have learned that the growth energy depends on substances that are present in the pericellular fluid and not on the energy derived from the ovum. As long as the cells are supplied with these substances, they exhibit an unlimited power of proliferation. The pericellular fluid of the adult organism does not contain growth-promoting substances in any noteworthy quantity. It is shown recently by Kiær*), that adult tissue cells and fibroblasts have the power of unlimited multiplication *in vitro*, when cultivated in media containing embryonic tissue juice. The tissue cells from the adult organism are able to produce unlimited amounts of living protoplasm when they are fed on embryonic tissue juice. In case of pathological processes, trephones are secreted by leucocytes or set free by dead muscle or gland tissue. As soon as the concentration of tre-

*) Personal communication.

phones increases in the pericellular fluid, the regeneration of tissue cells begins.

These facts help us to understand better the mechanism of repair in the organism. From numerous experiments Carrel has shown, that an aseptic wound made on an adult animal begins to cicatrize after two or three days.

If regeneration is a direct consequence of the loss of tissue, the healing of a wound, perfectly protected against irritation from outside, should take place normally. Such wounds were found by Carrel not to heal. The regenerative stimulus does not depend upon the tissue tension according to Ribbert. If the wound, instead of being protected against external irritation, was covered with a slightly irritating dressing, such as turpentine, a few staphylococci or dry gauze, cicatrization commenced within the two days. The action of the irritants is explained by Carrel in the following way, that it brings about the invasion of leucocytes. The healing of the wound is therefore due to the trephones secreted by the invaded leucocytes. The irritant does not act on the tissue cells themselves as Virchow believed in his assumption of the initiation of cicatrization from an inflammatory irritant, but causes the embryonic substances of the organism to be brought to the wound in the form of leucocytes.

Carrel mentioned that regeneration in lower organisms may also be determined by certain cell secretions. Loeb³⁷⁰ has observed that in tubularia, endodermic cells gather at the end where a new polypus is about to form, and probably set free substances necessary for the proliferation of the cells. As mentioned before Haberlandt²⁴¹ has observed that substances secreted by the leptom cells in certain plant tissues initiates the cell division of the plant cells. He calls these substances "wound-hormones".

From these experiments we have learned about processes which play an important rôle for the cicatrization, regeneration, transplantation and related phenomena. We have

learned how important the condition of the milieu extérieure is for these processes. In the milieu intérieure i. e. the protoplasm in the organism as a whole, we have the theatre for processes of the greatest importance, but very difficult to tackle by experimental investigation at the present time. Probably the many products, that we call internal secretions are transported directly from protoplasm to protoplasm by means of the cellular anastomoses. This is as yet unknown.

4. MORPHOGENETICS.

The method of cultivating tissues outside the body has mainly been employed for the study of the morphology of tissue cells. Seen from the point of view of the morphologists, the technique of explantation of tissue cells is a convenient method for the study of the structure of the various cells.

Preparation of living tissue cells on a coverglass have, of course, greater advantages than the ordinary histological methods. The cells can be observed in a condition of actual life for long periods of time under various experimental conditions. At any time the life of the cells may be interrupted and beautiful specimens made of the cultures, and the same cells observed during life can now be studied in the permanent preparation. The methods which the morphologists use for explantation, usually allow the tissue cells to grow out on the cover glass in a unicellular layer, and this gives stained and permanent preparations unsurpassed in beauty.

The methods of tissue explantation have also disadvantages in the hands of the morphologists. Very often the morphologists are not physiologists enough to avoid conditions which are too artificial and erroneous conclusions are easily drawn. The methods generally employed by the morphologists are to be considered as conditions of survival rather than cultures, according to Carrel's definition. This con-

dition is of course far from the natural. The cells are observed in solutions which may be called protective, but do not contain any nutriment for the cells. Many degenerative phenomena will therefore be interpreted wrongly.

The architecture of the tissue cells in general has been studied *in vitro*, under normal and pathological conditions. Within the last few years mitochondrial structures of the cytoplasm have been investigated specially. Lewis and Lewis have developed a beautiful technique and thereby contributed particularly to the study of the mitochondrial apparatus.

W. H. Lewis ³⁵⁵ has investigated the intimate structures of the fibroblasts ^{360 a}, endothelial cells ^{360 1} and epithelial cells ³⁵³.

In his excellent work on the cytoplasmic structures, W. H. Lewis concludes, that the tissue cultures afford an extremely good method for observations upon the undisturbed cells as they live. He finds mitochondria in all the cells and they can be studied in the living unstained cell for hours. The mitochondria may be scattered throughout the cytoplasm or they may be located around the nucleus. They change their position all the time. During mitosis the mitochondria become more evenly scattered throughout the cytoplasm, except in the spindle area, where they are usually absent. All shapes of granules can be found, and any one type of mitochondria such as granule, rod or thread may at times change into other type or may fuse with another mitochondrion — or again it may divide into one or several mitochondria. They may change their shape according to the experimental conditions. They may vary from minute granules, short rods to long threads. The number of mitochondria in a single cell vary from two or three to more than two hundred. The number of mitochondria is not constant for the same kind of cell. If the mitochondria degenerate, the rods or threads fall apart to granules — later these granules become vesicles and then separate as a num-

ber of small granular rings. At the time of mitosis, about one-half the quantity of mitochondria is separated into each daughter cell by the plane of division. The mitochondria are extremely plastic bodies and react more rapidly than any other cell structure. Their shape is easily influenced by heat, carbon dioxide, acids, alkalies, fat solvents and potassium permanganate, or by changes in the osmotic pressure of the surrounding medium. The mitochondria are stained in the living cells by Janus green, but not by Nile blue B extra or brilliant cresyl blue 2 b except in the dead cell. There was no evidence of a direct relation between the mitochondria and the formation of either the fat droplets or the vacuoles.

The generation granules and vacuoles do not arise from the mitochondria (W. H. Lewis ^{360 a}). By the combination stain of neutral red and Janus black Nr. 2, the degeneration granules are stained bright red and the mitochondria blue-black. Certain fixatives, especially those containing acetic acids, destroy the mitochondria leaving the granules unaffected. Also Prigosen ⁴⁴² was able to distinguish between degeneration granules and mitochondria.

The pigment formation was studied in vitro by T. Smith ⁴⁷². He found that there was no connection between mitochondria and the pigment formation. He studied the pigment in the retinal epithelium of the chicken's eye and found that the pigment granules were usually plump rods, the length varying from one and a half to five times their width. The colour was mostly dark brown or black. Light and heat had a marked influence on the movement of the pigment, in form of acceleration. They moved with a swift and jerky motion. Smith observed also the pigment granules or rods in the culture fluid, — a thing which I can only confirm. I observed these long ago in the embryonic tissue juice, and thought at first that they were bacilli, — further examination proved however to me, that they were the pigment rods from the retina. They had precisely the

same form as tubercle bacilli and absorbed the ordinary bacterial stains. When I stained these with methylene blue, thinking they were genuine bacteria, they absorbed the stain readily, often a little irregularly, somewhat similarly to that of bac. diphteria, showing granules and cross-striation. I was unable to observe at that time if these bacilli-looking rods were able to multiply independently of the cells.

In this connection it must be mentioned here, that Wallin ⁵⁰⁵ claimed quite recently to have cultivated the mitochondria in a special culture medium independently of the cells. If this be true, the cell is no longer the elementary organism of the body, consequently the Altmann theory is proved.

According to Smith, he observed in the cultures of the retinal pigment cells, cultivated in Locke-Lewis solution that these free granules underwent Brownian motion. Each of the cultures observed contained also a certain number of blood cells carried over with the explant. The red blood cells (nucleated) showed no tendency to take up the loose granules. The elasmotocytes did it so readily that they often became quite black with granules. Some of the connective tissue cells also took up the pigment — but not so readily as the elasmotocytes. This is a fact which is rather important to know because of the precautions to be taken when further studies are to be undertaken on the question of pigment formation. I myself have observed that the epithelium from the iris produced pigment, but only when the cells were allowed to remain in the same culture medium for more than 3—4 days. In the ordinary cultures, transferred every second day, no pigment formation was observed. Recently Ebeling ¹⁶⁵ gave a report of an 18 months old strain of iris epithelium in which report he claimed that the strain still produces masses of pigment. One has to be extremely careful in the conclusion that pigment formation actually takes place, when we consider the

presence of pigment rods and granules all the time in the culture medium.

Smith observed that the pigment rods, taken up by the fibroblasts, appeared to swell up and there was an actual decrease in colour. Such decolourization did not take place in the granules of the pigment epithelium. The pigment granules took up the neutral red in the cultures and the stain remained there even after fixation in Zenker's fluid.

Smith found that if he stained the cells with the combination stain of neutral red and Janus green the mitochondria stained bright blue with Janus green and the pigment granules took up the red. This differential stain between mitochondria and the degeneration granules and pigment has been used also by Cowdry¹⁴¹ and Lewis and Lewis³⁵⁵. Smith did not find that the pigment granules were extruded from the nucleus. There was no evidence that the mitochondria changed into pigment. The pigment granules arise and develop in the cytoplasm of the cell. Small colourless granules were seen in the cytoplasm of the cells, cultivated 12 hours; these granules increased in size, number and colour until they became rod-shaped and black of colour. The formation could be distinguished in two stages: a) the formation of a colourless chromogen and b) the production of colour in the chromogen.

Matsumoto⁴⁰⁹ investigated the granules, vacuoles and mitochondria in the sympathetic nerve fibres. He found that there was not a regular distribution of the mitochondria in the fibres or nerve end. The finding of this would of course be expected if the mitochondria gave rise to the neurofibrillae, such as has been claimed by several investigators.

The structure of certain male germ cells has been studied by Chambers¹¹⁶, Goldschmidt²²³, and M. R. Lewis³⁵⁹.

Experimentally the vacuolation and degeneration of the tissue cells have been studied. The introduction of bacillus typhosus in the hanging drop of the tissue cultures resulted in rapid vacuolation of the cells according to M. R. Lewis³⁴³). The effect of certain strong oxidizing agents as potassium permanganate on mesenchyme cells in tissue cultures was tried by M. R. Lewis^{360 e}.

The formation of fat droplets in explanted cells and the presence of glycogen in certain tissues under various conditions, were studied by M. R. Lewis^{340, 345}).

In certain degenerating mesenchyme cells, cultivated in vitro, W. H. Lewis^{360 c}) observed rather often giant centrospheres develop around the centriole. Just before the appearance of the centrosphere the centriole is surrounded by degeneration granules and vacuoles. The mitochondria become orientated about the centriole and centrosphere.

These giant centrospheres are interesting because they are claimed by Lewis to be identical with the cancer cell conclusions (P l i m m e r s bodies, cancer parasites). B o r r e l¹⁵) thought that these bodies found in cancer cells were the centrosomes of normal cells.

According to Lewis it seems surprising to find an enlargement of the centrospheres common to such different types of cells as epithelium and embryonic mesenchyme. It is probable that the fundamental metabolic processes are similar.

The giant centrospheres occurred in degenerating mesenchyme cells in the tissue culture, where, as Lewis says, we can to a certain extent control the environment; therefore the solution of this question is probably within the limits of experimentation. — The cultures were made in the usual way, and the factors producing this type of degeneration are not yet known. The illustrations in Lewis's article demonstrating this are extremely beautiful.

Another form of degeneration is the giant cells, the genesis of which is still unknown. Already from the very early days of tissue cultivation Lambert²⁸⁸ has studied the formation of the foreign body giant cells in vitro. He introduced *Lycopodium* spores into cultures of spleen. He also observed that other foreign bodies, such as cotton threads or the coverglass itself initiated the formation of giant cells. I myself* found in old degenerated cultures of epithelium giant cells with about 50 nuclei.

There has been much discussion as to the mechanism of the formation of giant cells. The general assumption of the origin of the giant cells is that of the fusion of few or several large mononuclear wandering cells. The other possibility of origin for the giant cells is that the nucleus divides without division of the cytoplasm. W. H. Lewis and Webster³⁶² have observed one clear case where the nucleus divided amitotic without any division of the cytoplasm. They also concluded that the giant cells seemed to be formed, for the most part, within the explant and to migrate out after their formation.

The origin and formation of the various intercellular substances has been studied by means of tissue cultivation. The hyaline substance of the cartilage, the bone formation, the enamel of the teeth, the fibrous fibrillae of the connective tissues and so forth will find an useful method in explantation.

Much discussed is the origin of the connective tissue fibres. The study of fixed and stained preparations of tissues has so far failed to decide the origin of the connective tissue fibrillae. It is still an open question, whether the fibrillae arise within the fibroblasts or from an intercellular substance.

*) Fischer, A. Unpublished experiments.

It is of course not in place here to go into the literature of the origin of the connective tissue fibres, but only to refer to some of the most important papers dealing with the problem from the point of view of tissue explanation.

One would think, that the modern technique of cultivating tissue cells *in vitro*, would be just the method for the solution of the problem. It is to-day still an open question, whether the fibrillæ are intra- or extracellular products.

Baitsell ⁷⁾ has shown in his beautiful work, that it is the fibrin network in the plasma clot which is transformed into fibrillæ under the influence of various mechanical factors. The main conclusion of Baitsell is that this transformation of the fibrin-network into connective tissue fibres, can take place without the presence of any living or dead tissue cells.

Harrison ²⁵¹⁾ has shown that it is absolutely necessary for the tissue cells to have a frame work or supporting apparatus for the growth and movements of the cells. This has already been claimed by Leo Loeb ³⁸⁰⁾ long ago, when he termed the phenomenon *stereotropismus*. Any one who has worked a little with tissue explants will soon have realized that tissue cells die very rapidly when placed in a liquid medium.

Baitsell ⁷⁾ shows that frequently a definite reaction occurs when a piece of tissue is placed in the plasma. Shortly after the tissue is placed therein, fibrillæ, resembling the connective tissue fibrillæ, are formed, which to begin with are quite small. By and by the fibres increase in size and number, and branch in all directions. The fibres are most solid near the tissue and from here they branch out into the plasma in all possible directions. Certain tissues give rise to fibrillæ much more quickly than others; spleen tissue develops fibrillæ very rapidly. These experiments seem to prove that the formation of the fibrillæ does not take place as an outgrowth from the tissue itself, but

that it is a transformation of the fibrin-net in the plasma clot. When the cultures get older, the entire plasma is transformed to thick, reticular and branching fibrillæ.

Serum preparations always failed to show any fibre formation, which indicates that the fibres are formed by a transformation of the fibrin-net and not as an outgrowth of the embedded tissue. My own experiments on the cultivation of complex tissues in serum seem to confirm this.

If tissue juice is added to the plasma, but no tissues, no fibres are formed. In other experiments Baitsell added to the plasma dead blood cells and in other experiments living blood cells or starch grains. In the plasma, to which living blood cells were added fibres were formed — but not in the plasma containing the dead cells or the starch grains. Baitsell found that a mere manipulation of the plasma with the preparation needles caused the formation of fibrillæ. This indicates that mechanical factors play an important rôle in the formation of fibrillæ.

In these studies of the transformation of the clot in tissue cultures, living tissues were present and although these experiments indicate that the formation of the fibres was not due to an intracellular action, the question could possibly be solved by the elimination of the living tissues from the plasma. Baitsell shows therefore in a very striking way that the plasma clot can be transformed to a new fibrous tissue, without the presence of any tissues, but under the influence of various mechanical factors.

A typical clot of plasma, as it can be observed with the ultramicroscope, is a network of connecting filaments, a crystalline gel. The coagulation as it is observed through the ultramicroscope, is beautifully described by Baitsell and accompanied with photographs. By pressing the coverglass against the fibrin clot during the coagulation for a short time, big consolidated fibrillæ are formed which look exactly like these found in vivo. If the fibrin clot is exposed to a pull, one will see fibrillæ in the direction

of the force; these fibrillæ increasing in length and diameter. The fibrillæ are located in the direction of the line of forces of the clot. If the clot is suspended in a moist chamber, the fibrillæ will have the direction of the gravity.

By the histological examination of the artificial fibrous tissue, it was shown, that there was no difference between those and the wavy bundles of fibrous tissue *in vivo*. They were stained in exactly the same way by Mallory's connective tissue stain. The illustrations in the papers of Baitsell are beautiful and convince us that the fibrous tissue artificially produced is identical with that *in vivo*.

It is of course well known that the supporting structures of the organism, are laid down in exact correspondance to the definite stresses; it is therefore concluded that the various mechanical factors are introduced during development, such as has been described by Baitsell to be the case with the plasma clot.

The other point of view as to the origin of the fibrous tissue fibrillæ, the intracellular origin, which standpoint is taken from experiments on tissue *in vitro*, is represented by M. R. Lewis³³⁹). The fibroblasts were cultivated according to M. R. Lewis, in fibrin-free media, and on the coverglass as support for the cells. The cultures of fibroblasts grow then in a way similar to endothelium and by special preparation (nitrate of silver and vapour of osmic acid) demonstrate the connective tissue fibrillæ. M. R. Lewis was able to observe mitochondria in the finest fibrillæ, a fact which speaks clearly for the cellular origin of the fibrils observed by Lewis. Probably the fibrillæ of Baitsell and the fibrillæ of Lewis are two different things, which both occur *in vivo*.

I myself am most inclined to assume that the Baitsell theory is the most correct. Both kinds of fibrillæ do exist in the body. Those of Lewis are probably the syndesmotie fibrillæ connecting the tissue cells in general and which play the important rôle in the function of the tissues.

and those of Baitsell are the connective tissue fibres as such.

The fibrillæ described by Lewis as containing mitochondria are the long delicate protoplasmic connections between the tissue cells, fibroblasts and epithelial cells, and they play quite another rôle for the cells than do the connective tissue fibrillæ according to Baitsell: the latter are the actual supporting apparatus of the tissue cells in vivo and a product of the coagulable substances of the humors.

According to M. R. Lewis she never observed the mitochondria to fuse into strands or become arranged in rows to form connective tissue fibrills such as several investigators have stated.

The formation of intercellular substance of cartilage can probably successfully be studied in vitro. Carrel and Burrows⁷⁴ have cultivated conjugal cartilage. They observed that the cartilage grew in the same culture and increased very much in size. They did not observe any disintegration of the hyaline substance in the way Fischer¹⁹⁴ has described it, as being the case with the cartilage from the eyeball of the chicken. I never observed any formation of hyaline substance in vitro. A slight variation in the composition of the culture medium would probably cause the cartilage cells to form intercellular substance; it is, however, still an unknown field. It is quite remarkable that the cartilage from the *pars cartilago sclerae* of the chicken eye, disintegrates entirely when embedded in the plasma medium and the cells die, but as soon as it is placed on the free surface of the clot, it commences to grow and the cells multiply. This fact seems to indicate that the physical forces prevailing on the surface of the clot is a *conditio sine qua non* for the growth of the cartilage cells in vitro.

Concerning the investigation of the various bone-substances and the bone-forming elements in vitro very little or nothing at all has been published so far.

Using tissue cultivation as a morphological and anatomical method, it is still greatly limited by uncertainty in the classification of the many cell types. As we have learned now, the shape of the cells cannot be employed in the classification. The morphology of the tissue cells may change according to the mechanical conditions of the culture medium (Uhlenhuth¹⁴⁸). Epithelial cells in vitro may appear polygonal, squamous, thin and elongated, fusiform like fibroblasts and so on. The only and best way of distinguishing between the various cells in vitro, is, as suggested by Congdon¹⁴³ to trace the outgrowth back directly to their source in the parent tissue. For this purpose sectioned cultures are necessary. The interrelation and the structures of the tissues generally disappear a short time after explantation. Tubules can be seen to grow out from kidney to begin with or solid processes may grow as a loose mesh (Lewis and Lewis³⁵³). Most explants contain several types of cells that migrate out into the medium, some of which are rather easy to identify.

Nerve fibres from the central nervous system have been observed by Harrison^{246, 249} in his pioneer work, where he showed that the regeneration of the axis cylinder is a kind of protoplasmic movement, and that from the neuroblasts of His, hyaline threads grew out which increased in length by means of amoeboid movements. Later Burrows²⁷, Ingebrigtsen²⁷¹, Levi³²⁹, Lewis and Lewis³⁵² have observed the same thing. Matsumoto described the growth of sympathetic nerves. Ingebrigtsen²⁷² studied the degeneration processes of the axis cylinder in vitro. Marinesco and Minea⁴⁰¹⁻⁴⁰⁶ investigated in large dishes the growth of the axis cylinders from spinal ganglia of young cats and rabbits in plasma.

Endodermal membranes from the cells lining the alimentary tract and allantois were studied by Lewis and Lewis³⁵⁰ and Lambert²⁸⁹. The identification of these

cells and tissues was finally made by Lewis and Lewis³⁵³.

Ectoderm from skin was studied by Carrel and Burrows⁷⁴, Lambert²⁸⁹, Uhlenhuth¹⁹⁷, Ruth¹⁶¹, Holmes²⁶³, Oppel¹³¹, Matsumoto¹⁰⁸; from the amnion by Lewis and Lewis³⁵³; from the retina by Uhlenhuth¹⁹⁷, Luna³⁸⁵, Smith¹⁷², Fischer²⁰⁰, and from the iris by Fischer¹⁹², Ebeling and Fischer¹⁶⁷, Ebeling¹⁶⁵.

Liver cells also form membranes similar to the entoderm and were described by Lynch³⁹⁰. The renal epithelium from the tubules also grows out in the form of membranes or sheets (Lewis and Lewis³⁵³, and sometimes as tubules (Champy¹³⁰); the thyroid gland cells grow out either as tubules or membranes (Carrel and Burrows⁷⁴. Recently Ebeling*) claimed to have cultivated the thyroid gland cells pure for a long period of time, which were producing colloid substance all the time in vitro.

Blood cells and wandering cells from the spleen have been studied by Carrel and Ebeling⁹⁶, Foote²¹⁶, Maximoff¹¹⁰; bone marrow cells by Carrel and Burrows⁷⁴, Erdmann¹⁷²; Lymph nodes by Lewis and Webster³⁶³; Thymus by Pappenheimer⁴³³.

Skeletal muscle has been beautifully described by M. R. Lewis³⁵⁶ and Lewis³⁵⁸). They suppose that the cross striated muscle fibres grow better in Locke's solution than in any other media, since among the numerous contributions to tissue culture, so little has been said about cross striated muscle by other observers who have used plasma media. Lewis and Lewis obtained such abundant outgrowth of muscle in their cultures. They describe that the muscle fibres were able to contract rhythmically for long periods of time. It was observed that there is a marked tendency for anastomosis and fusion of muscle

*) Ebeling, A. H. Personal communication.

buds either directly or by branches. The outgrowth of muscle resembles very much that of nerves in tissue culture. There is one capital difference, namely that the nerve outgrowth is entirely without nuclei, while the muscle fibre contains many nuclei both in the protoplasmic buds and in the connecting fibres.

Premuscle was studied by Congdon ¹⁴³. Heart muscle has been studied by Burrows ^{26, 29}, Congdon ¹⁴³, Levi ^{328, 333} and Olivo ^{427, 428}.

Smooth muscle, rather the ordinary mesenchyme, has been studied by Lewis and Lewis ³⁵⁸, M. R. Lewis ³⁴³. The contraction of the smooth muscle cell was studied by M. R. Lewis ³⁵⁷.

The connective tissue cells from adults have been beautifully described by Maximow ⁴¹⁰.

The endothelium was investigated by Carrel and Burrows ⁷⁴ and W. H. Lewis ^{360 f}.

The cartilage was studied first by Carrel and Burrows ⁷⁴ and later in pure permanent strains by Fischer ¹⁹¹.

VII.

TISSUE CULTURE AS A PATHOLOGICAL METHOD.

1. Studies in Immunity and Related Phenomena.

Most serologists assume, I believe, that the hematopoietic apparatus is the place where the antibodies are manufactured. Already in 1901 Romer⁴⁶⁵⁾ suggested that the lymphoid system was not the only place where antibodies were produced. By injecting abrin in conjunctiva of a rabbit immunity could be obtained. V. Dungern⁴⁵⁸⁾ was able also to obtain local immunity and he concludes that the production of antibodies is not reserved to special cells or organs, but that all tissue cells contribute.

As early as in 1912 Carrel and Ingebrigtsen³⁹⁰⁾ demonstrated that tissue cells cultivated outside the body had retained their property of reacting antigens by producing antibodies. They cultivated guinea pig bone marrow and lymph glands in guinea pig plasma. Goat blood was used as an antigen, because it was not at all hemolysed by guinea pig serum. The cultures of bone marrow containing the goat blood, and their controls were incubated for five days. Hereafter the presence of hemolysins was determined in the fluid extracted from the culture medium.

The extract from the cultures was mixed with a certain suspension of blood corpuscles and the hemolytic power investigated in the usual way.

The fluid of the cultures which contained goat blood was now able to hemolyse markedly goat red blood cells.

and the serum from the control cultures did not possess any activity.

It was observed that the guinea pig leucocytes took up the goat red blood corpuscles by phagocytosis.

The nature of the hemolysins was also investigated. The fluid hemolysed goat red blood cells without the addition of complement. After having been heated to 56° C. for half an hour it lost its hemolytic power. Reactivation could be established by adding complement. It was also found that amboceptor in the fluid from the cultures, could be adsorbed by goat red blood corpuscles in 1 hour at 0° C. According to these properties observed for the hemolysins produced in vitro, Carrel and Ingebrigtsen³⁰ concluded that tissues living outside of the organism react against an antigen by the production of an antibody.

Later Reiter⁴⁴⁸ has worked with the production of antibodies partly in vitro by using the modified technique of Lüdke³⁸⁷; the technique used by Reiter cannot be called tissue cultivation at all. He worked with entire organs from animals which previously had been injected with bacteria cultures as an antigen. Reiter observed that the production of antibodies began directly after the injection of the antigen, and he confirms the assumption of Wassermann, Pfeiffer and Deutsch that the antibodies are essentially a product of the hematopoietic organs.

The effect of specific hemolysins of living tissues in cultures has been tried by Hadda and Rosenthal²⁴¹. Their intention with the experiments was to investigate the effect of cytotoxins on tissue cells. This paper will therefore be discussed when the cytotoxic immunity and cytotoxins in general will be encountered.

Lately I have^{193, 196} studied the action of a small amount of an antigen on the rate of proliferation of fibroblasts in vitro. My intention with the experiments was to learn whether it was possible to have a strain of fibroblasts immunized against a foreign protein.

The technique was as follows: Cultures of fibroblasts belonging to a 9 years old strain (Carrel and Ebeling¹⁶⁴) were used. The preparation of the cultures was made in the usual way and the rate of growth measured by projection of the area of growth. The antigen used was human ascitic fluid or dog serum.

The culture selected for an experiment was cut into two equal parts. The one of the fragments was cultivated in a medium consisting of equal volumes of chicken plasma and fresh embryonic tissue juice. The other fragment was placed in a medium composed of equal volumes of plasma and embryonic tissue juice to which had previously been added the foreign protein in such a proportion that one volume of chicken plasma and one volume of the antigen-embryonic juice mixture would give the desired concentration.

These two cultures or strains were now kept separately, taking care that the control culture was not contaminated by foreign protein from knives or needles. When transferred to a new medium, each of the two cultures was divided. One half culture was used for the continuation of the experiment, and the other half was discarded, or used for testing how the cultures would act when exposed to the foreign protein in a high concentration. It is necessary to divide the culture every 48 hours, otherwise the central portion of the culture gets thick, badly nourished and finally necrotic.

In other words, one fragment was cultivated in a medium without antigen (control), the other fragment in a medium with antigen (experiment). After 48 hours incubation, both cultures, the experiment and the control, were divided into two. One-half of the experimental culture was transferred to a fresh medium with antigen and one-half of the control culture to a fresh medium without antigen. Both the experimental and the control strain were cultivated for a long period of time. The remaining halves of the ex-

perimental and control cultures were used as tests for measuring the resistance gained by the immunized tissue at a given time during the experiment. The test was carried out in such a way that the remaining halves of the experimental and control cultures were placed in a culture medium containing the antigen in a high concentration, 50–66 per cent, which was found to have a marked inhibiting action on the growth of normal tissues. If the tissue grown in a medium containing a small amount of antigen did not constantly become immunized, its rate of growth would be as low as that of the control tissue, when both were exposed to a high concentration of the antigen. Therefore, the quotient of the rate of growth of the experimental subculture (immunized strain in the high concentration of antigen divided by the rate of growth of the control subculture (non-immunized strain in the high concentration of antigen, expressed the degree of resistance or immunization. If both immunized and non-immunized strains, for instance, grew at the same rate in a high concentration of the toxic antigen, the quotient would be 1, and no immunization had taken place. If the immunized strain grew more actively than the non-immunized strain, the quotient would be higher than 1, and express the degree of resistance.

It was observed that the rate of growth of the two strains during 19 passages (about 10 days) was practically uniform, table 21 and fig. 16. The fluctuations of the rate of growth are due, to some extent to the slight changes in temperature of the incubator and similar periodical causes. In the beginning when the control showed a high rate of growth, the experiment showed a lower rate, and vice versa, but after about 8–10 passages both cultures showed fluctuations in the same direction.

Subcultures were made of the control (non-immunized strain) and both transferred into a medium composed of plasma, embryonic tissue juice, and the antigen. In one

series the subcultures were placed in media containing 50 % antigen ascitic fluid. This amount exerted a marked inhibiting action on the rate of growth of a normal culture of fibroblasts. The rate of growth of the control culture which had not been exposed to ascitic fluid decreased markedly or died, fig. 17. At passages Nos. 7, 10, 12, 11 and 17, half the tissue fragment was cultivated in a medium containing about 50 per cent of ascitic fluid, while the

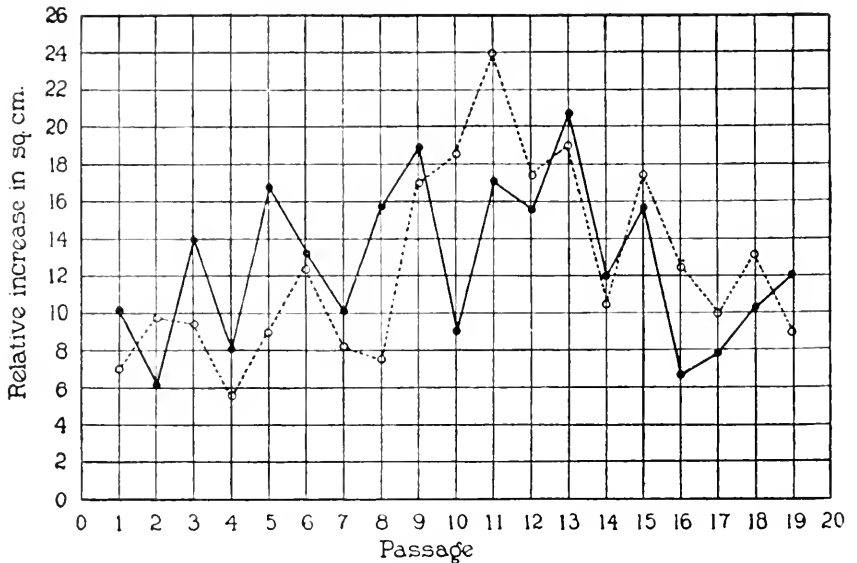


Fig. 46.

In all the figures the ordinates represent the relative increase in square centimeters and the abscissæ the number of passages at 48 hour intervals. The rate of growth of two cultures of fibroblasts, the control (dotted line) cultivated in the usual homogenic culture medium, the other (solid line) in the same medium containing 7 per cent ascitic fluid.

other half was used for the continuation of the strain. The dots A, B, C, D and E Fig. 17 represent the rate of growth of these subcultures during 18 hours. It can be seen that ascitic fluid in a concentration of 50 per cent has a marked inhibiting power on the rate of growth of a normal culture

of fibroblasts. The rate of growth of the fibroblasts which have been cultivated in a medium containing 7 per cent ascitic fluid is shown in fig. 48. At the same passages as in the control culture, namely 7, 10, 12, 14 and 17, subcultures were made in a medium containing about 50 per cent ascitic fluid and the dots A, B, C, D and E, indicate the

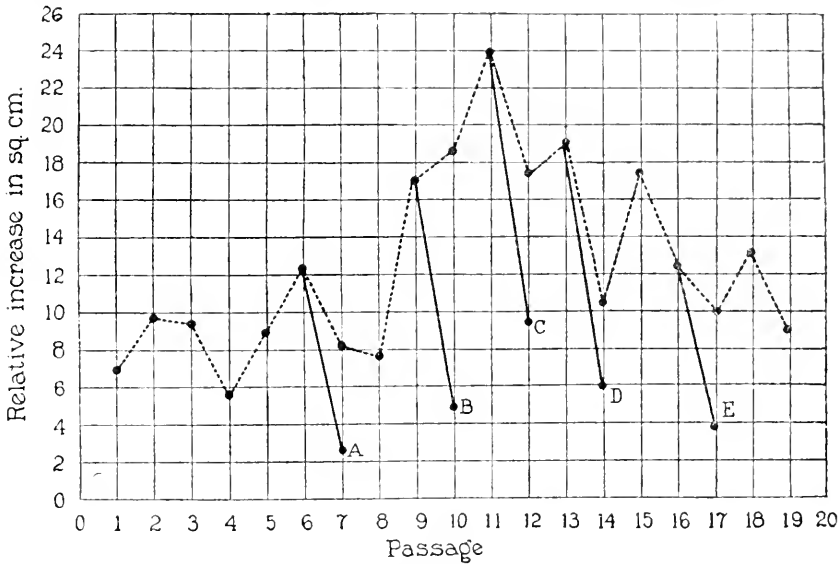


Fig. 47.

The rate of growth of the non-immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in homogenic culture medium. The solid lines (A, B, C, D and E) represent the rate of growth of the subcultures of the non-immunized strain in a medium containing 50 per cent ascitic fluid.

rate of growth. It may be seen that the rate of growth increased markedly instead of decreasing as in the control cultures. These two strains were no longer identical. There was always a sharp difference in their response to the same amount of the same foreign protein. It was certain, that the one of the strains had become adapted to the presence of the foreign protein, while the other was still influenced by its inhibiting power.

Table 21.

Rate of Growth of the Immunized and Non-Immunized Strains of Fibroblasts for a Period of Nineteen Passages.

Culture No.	Pas- sage No.	Relative increase.		Relative increase of subcultures in 50 per cent ascitic fluid.		Ratio $\left(\frac{E}{C}\right)$
		Control; homogenic medium.	Experiment; homogenic medium with 7 per cent ascitic fluid	Control.	Experi- ment	
537-1, 537-2	1	7.0	10.2			
549, 550	2	9.8	6.2			
559, 560-1	3	9.4	14.0			
571, 572	4	5.6	8.2			
585, 586	5	9.0	16.8			
599, 600	6	12.4	13.4			
610-1, 610-2)	7	8.2	10.2	2.8	13.8	4.9
611-1, 611-2)						
623, 624	8	7.6	15.8			
642, 643	9	17.0	19.0			
659-1, 659-2)	10	18.6	9.2	4.6	16.2	3.5
660-1, 660-2)						
672, 673	11	23.9	17.2			
686-1, 686-2)	12	17.4	15.6	9.4	23.2	2.4
687-1, 687-2)						
702, 703	13	19.0	20.8			
718-1, 718-2)	14	10.4	12.0	6.0	23.0	3.4
719-1, 719-2)						
733, 734	15	17.4	15.8			
749, 750	16	12.4	6.8			
760-1, 760-2)	17	10.0	8.0	3.8	8.4	2.2
761-1, 761-2)						
775, 776	18	13.2	10.4			
799, 800	19	9.0	12.2			

Peculiar in this experiment, is the increase in rate of growth of the immunized strain, instead of what was to be expected, that the rate of growth of this strain should be unaffected. It was, however, too constant a phenomenon

to be an accidental error, and the phenomenon may be related to some kind of anaphylactic process. Further experiments of this kind may probably throw some light upon the mysteries of anaphylaxis and anyway lead to interesting observations.

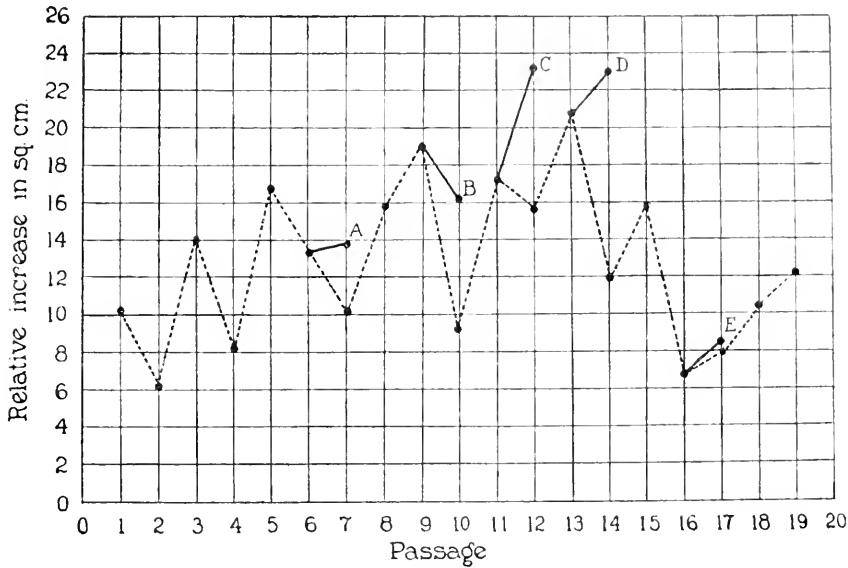


Fig. 48.

The rate of growth of the immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in the culture medium containing 7 per cent ascitic fluid. The solid lines (A, B, C, D and E) represent the rate of growth of the subcultures of the immunized strain in a medium containing 50 per cent ascitic fluid.

The rate of growth of the immunized strain in the high concentration of antigen, divided by the rate of growth of the non-immunized strain in the high concentration, expresses the degree of immunization or resistance to the toxic action of the foreign protein. Fig. 49 is a curve which indicates the degree of immunization at given periods during the long experiment of the 17 passages. The curve rises

10-12 days after the beginning of the experiments. Afterwards, the curve declines slowly. This and some other experiments (fig. 50) remind one of some carried out by Jørgensen and Madsen²⁸⁰, in which they show the

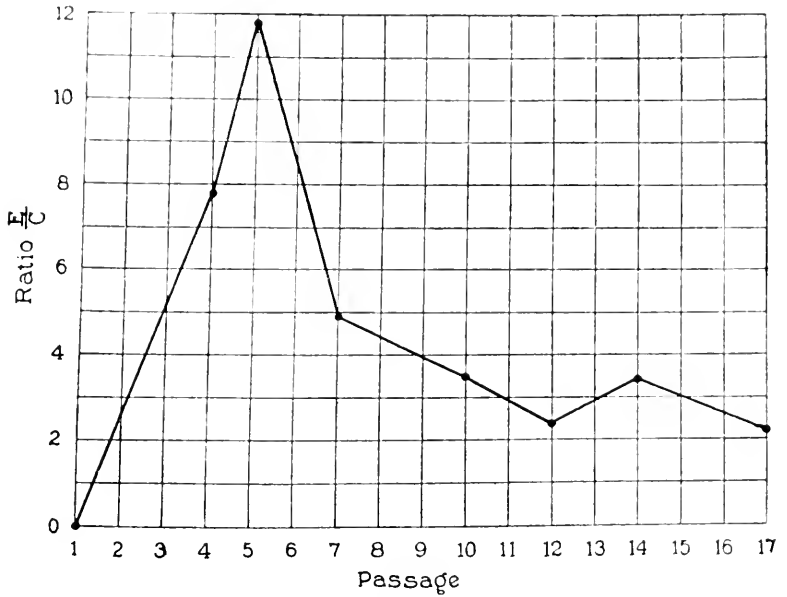


Fig. 49.

Curve showing the variations of the degree of immunization of fibroblasts through seventeen passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

amount of agglutinin produced by a goat under the influence of continued injections of the antigen, cholera bacilli: the conditions being almost the same as in my own experiment.

Table 22.

Immunization of Fibroblasts by Dog Serum in 1,4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1118 -2, 1119 -2	2	0,92
1257-2, 1258-2	3	1,14
1602 -2, 1603 -2	4	1,06
1670 -2, 1671 -2	5	1,23
1637 -2, 1638 -2	6	2,13
1456-2, 1457-2	8	1,90
1908 -2, 1909 -2	9	1,17

Table 23.

Immunization of Fibroblasts by Dog Serum in 4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio.			
		Immunized and non-immunized strains.	Non-immunized strain and its sub-culture.	Immunized strain and its sub-culture.	Subcultures of immunized and non-immunized strains.
1180, 1181	1	0.68			
1216-1, 1216-2)	2	1.90	0.43	0.36	0.86
1217-1, 1217-2)	3	0.51	0.50	0.30	0.60
1255-1, 1255-2)	4	1.03			
1256-1, 1256-2)	5	1.17	0.25	0.59	2.40
1289, 1290	6	2.00			
1326-1, 1326-2)	7	2.80	0.30	0.41	1.40
1327-1, 1327-2)	8	0.30			
1361, 1362					
1397-1, 1397-2)					
1398-1, 1398-2)					
1452, 1453					

Table 24.

Immunization of Fibroblasts by Dog Serum in 8 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1491 2, 1492 - 2	2	1.95
1713 2, 1714 - 2	3	1.25
1489 - 2, 1490 2	4	0.60
1538 - 2, 1539 2	5	0.25
1094 2, 1095 - 2	6	0.30
1170 - 2, 1171 - 2	8	0.45

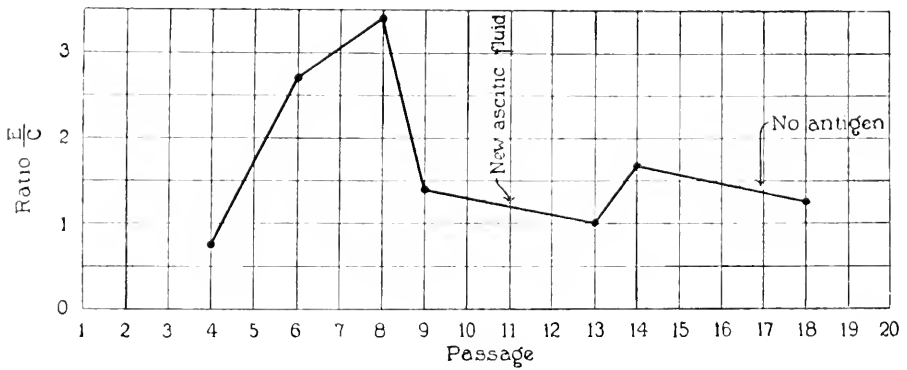


Fig. 50.

Curve showing the degree of immunization of fibroblast through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

In other experiments dog serum was used as antigen and similar results were obtained. The dog serum was found to be a good deal more toxic against chicken fibroblasts than the human ascitic fluid.

An investigation was made as to the concentration of the antigen used and the degree of immunization of a strain

of fibroblasts. Dog sera were used which had a marked toxic effect on the fibroblasts. When 1.1 per cent antigen was added to the culture medium as a small daily dose, the maximum degree of immunization or resistance was reached at about the 6-8 passage, table 22 and fig. 51. When 1 per cent antigen was added to the culture medium, the maximum degree of resistance was reached at the 5th passage, table 23. When 8 per cent antigen was used, the maximum degree of immunization was reached at the second passage already, fig. 52, table 24. A high concentration of antigen caused a quick rise of resistance which

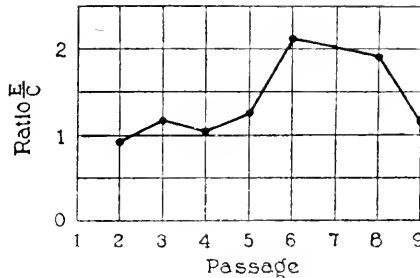


Fig. 51.

Curve showing the variations in the degree of immunization of fibroblasts through nine passages. Dog serum was used as antigen in a concentration of 1.4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

lasted only a short time, whereas the maximum degree of immunization was reached relatively late and lasted long, when a small amount of antigen was used. This is also found by earlier experiments *in vivo*, Thorborg¹⁹⁶.

The experiments here are of course only to be considered as a kind of exploration in this field of investigation. The results anyway show clearly that fibroblasts respond also to the presence of a foreign protein in the medium and become immunized and there is doubtlessly a relation be-

tween the amount of antigen, the time of the appearance of the immunization and its duration.

Whether the phenomenon described here really is an immune process inasmuch as the fibroblasts actually produce antibodies, is of course not yet proved by these experiments. To settle the question it is necessary to demonstrate the presence of antibodies in the culture medium of the immunized strain. If the culture medium of a non-immunized strain is able to protect the latter from the toxic action of the antigen passive immunization it is proved

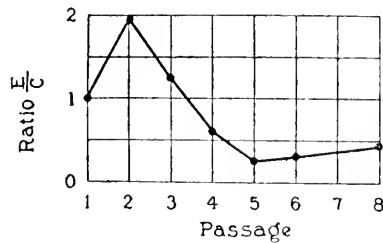


Fig. 52.

Curve showing the variations in the degree of immunization of fibroblasts in vitro through eight passages. Dog serum was used as antigen in a concentration of 8 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

that the acquired resistance is an actual process of immunization. This has not yet been done.

If there is a veritable immune process going on here, and not a simple adaption, which I do not believe, we have then a simple technique for making fundamental studies in this field of investigation and in clean-cut experiments much simpler than if we work with the complex organism. At the same time, as the tissue cells produce antibodies in vitro, we have in the rate of growth of the same tissue an indicator for the degree of immu-

nization. In the permutation of the various quotients of the immunized and non-immunized strain and their respective subcultures under various conditions, we are able to obtain all possible kinds of information as to the conditions of the strains during the process. We can, for instance, get information as to the action of the small amount of antigen on the immunized strain during the process of immunization. If the antigen in this low concentration should have any growth-inhibiting effect, the quotient of the rates of growth of the immunized strain and its control would be below 1. Fig. 53 represents such a curve, showing the

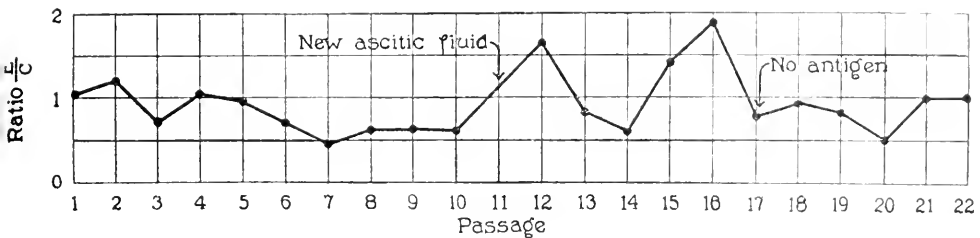


Fig. 53.

Curve showing the rate of growth of the immunized strain and its control through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of its control, and the abscissæ the number of passages.

effect of 7 per cent human ascitic fluid on a strain of fibroblasts during 22 passages. Fluctuations can be seen, the cause is as yet unknown, but generally taken, the index is about 1 all the time, which denotes that the action of 7 per cent human ascitic fluid has practically no inhibiting effect on the strain.

If the quotients of the rates of growth of both subcultures of the immunized strain in low and high concentration are figured out, and if the quotients of the rates of growth of both subcultures of the non-immunized strain, without an-

tigen, and in a high concentration of antigen are figured out, it can be seen that the immunized strain grows more rapidly than the non-immunized. Fig. 54, and fig. 55 illustrate this. These are examples of the mode of interpreting the experiments.

In order to demonstrate in a clear way the various quotients of rates of growth which I have mentioned here, it is simpler to look at the following diagram, fig. 56. From this it can be seen that A is a culture with a small amount of antigen. A_1 and A_2 show the same culture divided in halves. A_1 is carried on in a medium containing a small

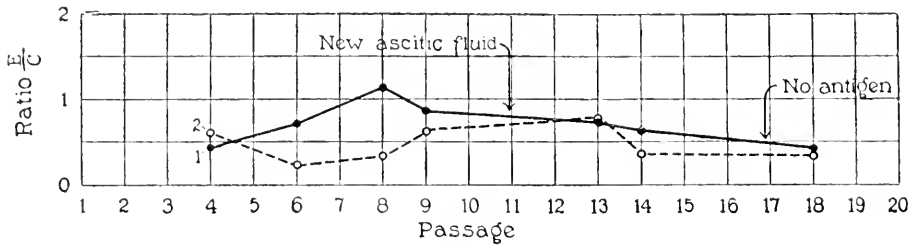


Fig. 54.

Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration of antigen. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in the ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rates of growth, and the abscissae the number of passages.

amount of antigen (the continuation of the experiment): A_2 subculture is transferred to a medium containing the antigen in a toxic concentration. B is the control culture. B_1 is carried on as B, the control culture continuation of the control, and B_2 is transferred to a medium containing the antigen in a toxic concentration.

Hereof follows that $\frac{A_2}{B_2}$ expresses the degree of immunization of A.

$\frac{A_1}{A_2}$ expresses the direct effect of antigen on the immunized fibroblasts.

$\frac{B_1}{B_2}$ expresses the effect of the toxic concentration of antigen on normal fibroblasts.

In addition to these experiments with the action of foreign protein on tissue cells in vitro, it must be mentioned that Carrel and Ebeling²⁷ found that casein inoculated into a culture of leucocytes brings about an immediate increase in the substances which promote the growth of fibroblasts and natural hemolysins. The cells respond imme-

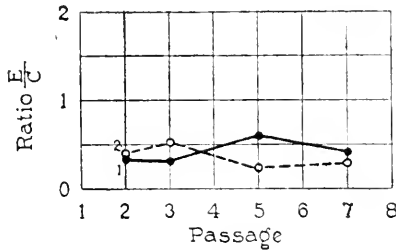


Fig. 55.

Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient or the rates of growth, and the abscissæ the number of passages.

diately and non-specifically to the foreign substances. These experiments by Carrel and Ebeling indicate a quite new field of investigation namely the therapeutic action of non-specific foreign proteins. This question would have an ideal method in the tissue cultivation.

The phenomenon of anaphylaxis has not been studied by means of tissue cultivation. Attempts have been made to investigate the cellular action under anaphylactic reactions by means of isolated organs, i. e. uterus, lungs, liver suspended in Ringer's fluid, Weil⁵⁴⁴⁻⁵⁴⁵, Manwaring and Crowe⁵⁴⁸⁻⁵⁴⁹. The tissue cultivation technique will

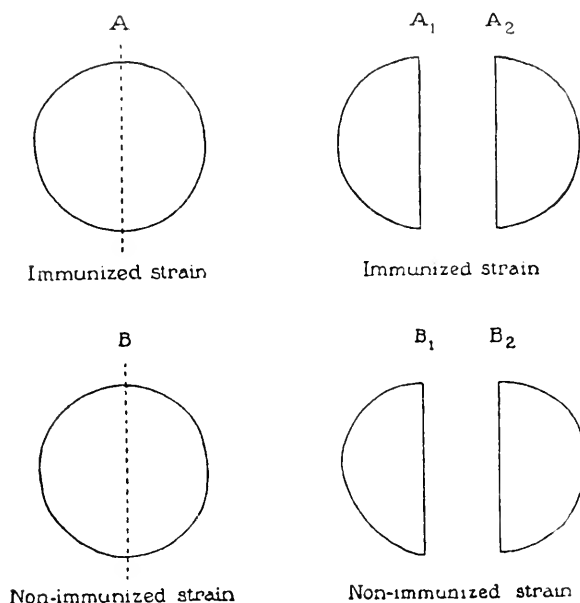


Fig. 56.

A is a culture with a small amount of antigen. *A*₁ and *A*₂ show the same culture divided in half. *A*₁ is carried on in a medium containing a small amount of antigen; *A*₂ (subculture) is transferred to a medium containing the antigen in a high concentration. *B* is the control culture. *B*₁ is carried on as *B*, the control culture, and *B*₂ is transferred to a medium containing the antigen in a high concentration. The quotient of *A*₂ divided by *B*₂ expresses the degree of immunization of *A*₁.

probably also here be of excellent help for the investigation of anaphylaxis. The stimulation of the rate of growth, as I found it for "immunized" fibroblasts when exposed to a

toxic concentration of the antigen, is doubtless a question of anaphylaxis. Further experiments should be taken up again.

Precipitin reactions have also been studied a little by the means of tissue cultivation. Przygódc⁴⁴⁵⁾.

2. *Tissue culture as a bacteriological method. Effect of bacteriotoxins on living tissue cells in vitro.*

The idea of studying the interaction of bacteria and living tissue cells in close proximity directly under the microscope in tissue cultures was put into practice by Smyth^{479) 480) 481)}. One would think that tissue cultures might be the most excellent experimental method for the study of various pathogenic bacteria. Undoubtedly the tissue cultures may, in many cases, be the culture method for living virus of varying nature, which hitherto have withstood all attempts at a successful cultivation in vitro such as poliomyelitis, vaccina, rabies, typhus fever and many others. The conditions within a tissue culture are very similar to those of the body, in any case in respect to the composition of the culture medium and many pathogenic bacteria, probably also these of the ultra visible may be cultivated successfully in that manner. The virus of the Peyton Rous chicken sarcoma, which must be considered as a ultravisible virus, can be cultivated in vitro after the method I have devised, and it is therefore the first filtrable virus which has been propagated indefinitely outside the body.

Smyth^{479) 480) 481) 482) 483)} studied the bac. typhi, bac. diphteria, bac. pseudo-diphteria, bac. coli, micrococcus aureus and bac. prodigiosus in cultures of both embryonic as well as adult tissues of chicken, mostly heart tissue. The author claims that the technique of tissue cultivation in vitro is a valuable addition to the methods of bacteriological study. He is absolutely right in this; it does not seem to be realized so far; possibly the use of so complex a technique frightens.

Smyth likewise tested the action of diphtheria toxin on the tissue growth in vitro. The technique he applied in 1911-15 was probably not the very best. He concluded that chicken plasma has a marked bactericidal action on the bac. typhi, which may, in some slight degree, be overcome by the presence of growing tissue, especially splenic tissue in the cultures.

On bac. dysenteriae the bactericidal action of chicken plasma is present, but much less markedly, and the same antagonistic action of the tissue was observed.

Chicken plasma has little or no bactericidal action on bac. coli.

On bac. diphtheriae chicken plasma is strongly bactericidal and the action is counteracted by the presence of the tissue in the culture.

Smyth⁴⁸² observed also the interesting fact, that many bacteria stimulated the proliferation of tissue cells. This I can fully confirm through occasional observations.

Most interesting are the observations of Smyth⁴⁸³ on the action of tubercle bacilli on various tissue cells grown in vitro. The growth of the fibroblasts and epithelial elements, the formation of the epitheloid cells and giant cells, the degeneration and disintegration of the tissue cells was beautifully observed.

3. *Specificity of the tissue cells. Cytotoxins.*

For the demonstration and the study of the specificity of cytotoxins, the tissue culture technique is a method which will admit of the direct observation of cells in an undisturbed state for a definite period of time. The tissue culture technique was primarily used for this particular study by Lambert and Hanes who perceived the importance of this investigation. The application of this technique is dependent upon the possibility of cultivating in vitro the various cells in pure strains. Since 1911, when Lambert and Hanes took up this study, the technique of cultivating

the tissue cells in vitro has progressed very greatly; nevertheless this particularly interesting and important field has not been studied practically since that time. To-day when it is possible to work with pure strains of several types of epithelium, fibroblasts, cartilage, leucocytes and sarcoma cells, the investigation of the cytotoxins should be taken up again.

Practically any tissue of the body has been studied in respect to their specific cytotoxins, obtained by injecting the various tissue cells into animals of a foreign species. Leucocytes has been studied by Metchnikoff ⁴¹⁴, Besredka ⁴¹⁵, liver by Delezenne ⁴⁴⁸, Deutsch ⁴⁴⁹, kidney by Lindemann ³⁶⁶, Nefedieff ⁴¹⁸, Bierry ⁴², Ascoli and Figari ⁵, pancreas by Surmont ⁴⁸⁹, adrenal by Bigart and Bernard ¹³, thyroid by Goutscharkow, Mankowski ³⁹⁷, heart muscle by Centanni ¹¹², ovary by Ceconi and Robecchi ¹¹¹, nerve tissue by Delezenne ⁴⁴⁸, Centanni ¹¹³, synectium by Ascoli ⁴, Liepmann ³⁶⁵.

In order to test the specificity of cytotoxins several methods have been used. Emulsions of cells have been exposed to the action of immune serum, and the lytic and agglutinative properties were observed. But the lytic and agglutinative properties cannot of course very well be indicatives of cytotoxic action, just as little as agglutinins of typhoid bacilli are indicatives of bactericidins. Therefore the technique generally used was to inject the immune serum into the animal body and use the functional or histological changes in various organs, corresponding to the supposed action of the immune serum, as a criterium for the specificity of the cytotoxins. To day the complement fixation would naturally be employed as the proper method. As the technique of making tissue cultures has greatly progressed since that time, we are now able to work with pure strains of the tissue cells, and are able, in a relatively short time, to get plenty of material of pure cells from cultures, enough for the injection into the animals.

Lambert and Hanes³¹⁶ and Lambert²⁹⁵ have mainly studied the cytotoxins of various malignant tissue cells. Rats were immunized by mouse sarcoma. In the plasma of the immune rats, bits of mouse sarcoma were cultivated. It was observed, that the sarcomatous tissue from the mouse remained quite inactive for a long time in some of the cultures and later it was noted that a few cells of irregular shape wandered out. In other cultures the tissue disintegrated markedly. These experiments indicated that the plasma from the immune rats was no longer a favorable medium for the cultivation of the mouse sarcoma, such as was the case when mouse sarcoma, was cultivated in plasma from a non-immunized rat. Several experiments of the same kind gave similar results. These experiments correspond very well to what is known from experiments *in vivo*. Mouse sarcoma inoculated into normal rats grows actively for 8–10 days, producing a nodule which is afterwards rapidly absorbed. In rats previously immunized, no tumor nodules are formed.

In other experiments guinea-pigs were immunized with either defibrinated blood or rat sarcoma. It was then noted that rat sarcoma cultivated in plasma from normal guinea-pigs, gave uniform and good cultures. The rat sarcoma in the plasma from the immune guinea-pigs, immunized with rat sarcoma, showed no activity whatever. Also these experiments showed that plasma from guinea-pigs previously treated with rat sarcoma was unsuitable for the growth of rat sarcoma. The cytotoxins produced by immunization of guinea-pigs with rat sarcoma is not specific, but is also produced when guinea-pigs were immunized with defibrinated rat blood.

Foot²¹⁵ has studied the cytotoxic action of the bone marrow immune plasma on bone marrow cells *in vitro*. He found that the bone marrow cells degenerated and died when cultivated in the immune plasma from a rabbit. He also observed a precipitin reaction in the cultures. Foot clai-

med further that the immune plasma from the rabbit against chicken bone marrow cells, also had a marked cytotoxic effect on the rabbit bone marrow cells.

All these interesting investigations ought to be again taken up since we are now able to master the technique of tissue cultivation and since now we know a good deal more of the action of heterologous culture media on the tissue cells.

It is difficult to find out what the general opinion is as to the existence of an organ specificity. Many investigators concluded from their experiments, especially from a study of the lesions found in animals, injected by "specific organ immune substance", that such a specificity can be demonstrated. According to Lambert²⁹⁵, Lüdke and Schüller³⁸⁸, they have described the production of a nephritis after the injection of the serum of rabbits immunized with dog kidney. Pearce¹³⁷ explains the various lesions following the injections of cytotoxic sera as a direct toxic action on certain parenchymatous cells, of the hemagglutinative and hemolytic properties of the serum, developing thrombosis with all its consequences. He does not believe at all in specific somatogenic cytotoxins.

Lambert²⁹⁵ claims in his paper on this subject that the problems can be solved from the methods of tissue cultivation, because here the active living cells may be exposed to the action of the immune sera, and under experimental conditions, factors such as agglutination, thrombosis and hemorrhage can be eliminated.

Lambert²⁹⁵ described the experiments he has undertaken with rat sarcoma and rat embryo skin, experiments which fully confirm what has been found earlier by other methods.

Guinea-pigs were inoculated subcutaneously with 0.15 gram rat sarcoma, and others with a similar amount of embryo skin. After the termination of the immunization, the animals were bled and the plasma obtained for the culture. Plasma from normal guinea-pigs were used as controls.

The embryo skin and rat sarcoma cultivated in the plasma from normal guinea-pigs, showed after 48 hours incubation a luxuriant growth in practically every preparation. The preparations containing the immune plasma were on the contrary much less active though the majority showed, especially on the third and fourth day, a fair out-wandering of cells. There was a definite difference in the behaviour of both kinds of tissue in the plasma from the two animals treated. The tissues, skin and sarcoma, both showed a more marked inhibition of growth in the plasma from the guinea-pig injected with sarcoma, than in the plasma from the guinea-pig injected with embryo skin.

By injecting some guinea-pigs with a much larger dose of rat sarcoma 0.65 gram and some with a similar amount of skin, the plasma had a marked increase of inhibiting action of the tissues. The plasma from the animal previously treated with a large quantity of embryo skin, was the most toxic for both sarcoma and skin. A perfect disintegration took place of the tissue explanted in this plasma.

These experiments showed that there was no evidence of even a relatively specific action of the cytotoxins produced, the plasma from each animal having an equally inhibiting effect on each kind of tissue. It was also observed that defibrinated rat blood could be used to immunize against both sarcoma and skin. Other experiments showed that it was not possible to obtain specific cytotoxins for either normal intestines or heart tissue of chicken.

Similar experiments to Lambert's have not been continued or taken up, since it is now possible to work with pure strains of certain types of tissue cells. Possibly the results will be different from those already reported, because earlier work was carried out with tissues containing all kinds of tissue elements.

4. Tumors.

Shortly after Carrel and Burrows had improved Harrison's technique of tissue explantation and extended

the method to the cultivation of other tissues than nervous fibres, and to comprehend tissues from warm blooded animals, and attempts were made to cultivate malignant tissue cells in vitro. The results of these experiments in general are few, and have been rather disappointing to most investigators. Most observations hitherto made on malignant tissue cells in vitro, have not been able to give any biological characteristics of these cells, which were not also found for normal tissue cells.

As early as in 1910 Carrel and Burrows ⁷⁶ issued the first reports about the cultivation of malignant tumor cells. They succeeded quickly in cultivating the Rous chicken sarcoma, the Ehrlich and Jensen sarcoma of the rat, a primary carcinoma of the breast of a dog ^{76) 77)}, and two human tumors, a sarcoma of the fibula and a carcinoma of the breast. Lambert and Hanes ^{306) 307) 309)} have cultivated rat and mouse sarcomata, and a mouse carcinoma.

Loose and Ebeling ³⁸³⁾ have cultivated human sarcomatous tissue in vitro. Recently Drew ¹⁵⁵⁾ has made a comparative study of normal and malignant tissue cells in vitro. His technique is that which only allows of a survival of the cells and is not a culture according to Carrel's definition.

As just stated, the malignant tumor cells in vitro have not manifested themselves in any striking and characteristic way. The only difference from that of normal tissue cells is that it proved to be technically more difficult to cultivate malignant cells in vitro than was found to be the case for normal tissue cells. So far, it has only now been managed to overcome the technical difficulties for the cultivation of the Rous chicken sarcoma indefinitely outside the body, in the same way as is the case with that of fibroblasts, epithelium, cartilage and lymphocytes. Probably other malignant tissue cells from other animals and even the human tumor cells may be cultivated in a similar way ^{199) 203)}.

Neither Lambert and Hanes³⁰⁹⁾, Carrel⁵⁶⁾, Carrel and Burrows, or Losee and Ebeling³⁸³⁾ observed any biological characteristics which were not also found for normal tissue cells. According to Lambert and Hanes, sarcomatous tissue grows as a type which may be regarded as characteristic for tissues of mesenchymal origin. The outgrowth of carcinoma cells assumes a sheet-like form, only one cell in thickness. This is characteristic for normal epithelium as well. Both sarcoma and carcinoma cells cultivated in vitro were found by Lambert and Hanes³⁰⁹⁾ rapidly to absorb carmin particles placed in the plasma.

All investigators agree as to the difficulties in cultivating the malignant tissue cells in vitro. Carrel⁵⁶⁾ stated that sarcomatous tissue grew as well during a few days as normal fibroblasts, but afterwards the rate of growth became less rapid and the tissue could not be kept alive for more than about two months. The life of the cultures of malignant tissue cells is short, and may be due to technical factors.

Losee and Ebeling³⁸³⁾ were able to keep the human sarcomatous tissue alive in cultures for 52 days. Kiær and Fischer*) kept a human glio-sarcoma in an active condition of life for more than two months.

The technique of cultivating the various tumor cells, varied according to the nature and origin of the tumors. For the cultivation of chicken tumors, the technique was simply exactly the same as used for the cultivation of normal tissues. Homogenic culture media could be used. The mouse and rat tumors were often cultivated in mixtures of chicken plasma and plasma or serum from the respective animal. Kiær and Fischer*) cultivated the human glio-sarcoma to begin with in a mixture of 20 per cent chicken plasma in extract of the sarcomatous tissue and human serum. Later, the same strain of sarcoma was cultivated in chicken

*) Kiær, S. and Fischer, A. Unpublished experiments.

*) Unpublished experiments.

embryonic tissue juice from young chicken embryos, for about a month. In other words the culture medium for the human sarcoma was entirely heterologous.

The human tumors cultivated by Carrel and Burrows⁷⁵, Losee and Ebeling³⁸³, were either cultivated in autogenic or homogenic plasma. Losee and Ebeling cultivated the human sarcomatous tissue in a medium composed of equal parts of normal human plasma and Ringer solution and varying the quantities of extract of fresh human fetal cadavers. The technique of preparing the cultures was the same as so often described, and it is not necessary to repeat it here.

All investigators agree that the malignant tumor cells liquefy the plasma medium so extensively that all further growth ceases and the tissue dies. It is probably the liquefaction which has complicated the cultivation of tumor cells. The liquefaction depends very much upon the nature of the plasma medium and the tumor cells. Human plasma is liquefied much easier than chicken plasma, for instance, and certain tumor tissues possess liquefying properties much greater than others. It seems to be very difficult to prevent liquefaction of the plasma clot. Recently Carrel⁶⁶ found that the addition of small amounts of serum and a trace of sodium linoleate to a fibrinogen-Thyrodé-medium, partly prevented the liquefaction of this medium when normal fibroblasts were cultivated in it. I have tried this modification myself in the cultures of sarcomatous tissue — but without success. The best results of that kind I obtained by reducing the amount of embryonic tissue juice in the culture medium.

Liquefaction of the plasma medium generally occurs after a certain amount of new growth has appeared, usually a few hours after the cultures are prepared or not until after 15–21 hours. As soon as liquefaction has taken place, all connection between the original explanted fragment and the new growth is broken off, and on account of the change

of the plasma medium from solid to a liquid, all further outgrowth from the fragment is impossible. When transferring the tissue from one culture to another, it is possible only to carry over the original fragment which floats in a lake of the liquefied plasma. After being brought to a fresh medium, the liquefaction recommences, and so on. Continued this way, it is clear that we do not even get a multiplication of the tissue cells, but on the contrary the tissue decreases in size and finally succumbs.

Recently I therefore developed a new method for the cultivation of malignant tumor cells, which proved to be very satisfactory. The idea of the method I conceived by considering the ability of the malignant tumor cells *in vivo* to invade the surrounding normal tissues and displace their cells. The question was, would the sarcoma cells, for instance, also invade normal tissues added to the cultures of sarcoma cells?

The Rous chicken sarcoma was used in the experiments, not because of the peculiarities of this tumor (infectious fowl tumor), but because it was a chicken tumor, which would be rather easy to cultivate in its homogenic culture media, and because the method of cultivating chicken tissues in general is so perfect and well organised.

The chicken sarcoma belonged to the type of polymorphous celled sarcoma. The grafts on chickens of all kinds had a take of about 100 per cent.

The sarcomatous tissue for cultivation was excised under ether anaesthesia from the tumor bearing animal, about 4—6 weeks after inoculation. Sometimes the tissue for the cultures was taken from the original tumor and sometimes the tissue was taken from metastasis in the lungs shortly after the spontaneous death of the animal.

The malignant tissue was cut into small pieces and placed in the culture medium, usually composed of two volumes of chicken plasma and a trace of freshly prepared embryonic tissue juice. The growth of the Rous chicken

sarcoma in this medium was the same as described earlier by Carrel and Burrows⁸⁷. The cells are mainly active amoeboid and wander far out in the plasmic medium. Already after a few hours cultivation, a very extensive liquefaction occurs and the migrated amoeboid cells can now be seen contracted as spherical bodies, floating around in a lake of liquefied plasma clot.

To the cultures of sarcomatous tissue were now added small bits of muscle, excised directly from normal adult chickens, or taken from fragments of muscle which had been stored in Ringer solution for a long time in the refrigerator. The small fragments of muscle, of about the sizes of the explained tissue itself, were placed almost in contact with the fragment of sarcoma in the culture medium.

The muscular tissue is taken from the chickens, from the muscles of the neck, when the chickens are bled to obtain the plasma. The muscle-fragment is quickly placed in a small jar with Ringer solution, and stored in the refrigerator. As often as it is to be used, it is taken out and cut into small pieces. These are generally embedded first in the usual culture medium by themselves and incubated for about 2—4 days and thus tested this way for infection and to see if any outgrowth from the muscles takes place. Not a single cell has been observed to grow out from such a piece of muscular tissue, stored in the ice box from 24 hours to one month.

The muscle used for the experiments is always selected to be of as pure muscular tissue as possible, without tendineæ or connective tissue. By storing the muscle-fragments for about 14 days in the refrigerator, all the cells have died and in any case are not able to grow in the cultures.

After having tested the fragments of normal muscular tissue in the way just described, they are cut out of the plasma clot, washed in Ringer solution for about half a minute and a single piece is placed together with the sarcomatous tissue, side by side in the same culture. When

the culture, containing the little fragment of normal muscular tissue and the sarcoma has to be renewed and transferred, the two fragments are picked up on the point of the needle or knife from the liquefied medium, washed in Ringer solution and replaced in a fresh culture medium. After a few passages, the two fragments generally adhere to each other and can be transferred as one piece.

After about 4–6 passages the muscular tissue is perfectly destroyed and a new piece must be added. Before the muscular tissue is destroyed and completely invaded by sarcoma cells, the total mass of tissue can be divided and subcultures made.

When a small fragment of the Rous chicken sarcoma is cultivated in the usual way in chicken plasma and embryonic tissue juice, an extensive liquefaction takes place in the surrounding medium within few hours, and the fragment is found after 24 hours to be floating in the medium surrounded by numerous spherical cells. After a few passages, the fragment becomes smaller and smaller, and dies. The liquefaction of the culture medium prevents actual increase of the mass and the tissue cannot be divided and the cultures multiplied, as can be done with the fibroblasts and epithelium. On adding a piece of solid muscular tissue to the sarcomatous tissue in the culture, the sarcoma cells are observed to migrate abundantly to the muscle before any liquefaction of the medium has taken place. After the liquefaction has begun, many cells have already migrated over in the muscle where they multiply very extensively, independently of the liquefaction going on.

After a few passages, sometimes already after the first passage, the normal muscle fragment and the sarcomatous tissue adhere to each other, so they can be transferred as one piece of tissue. After 2–4 passages, the muscular tissue is so invaded by sarcoma cells, that it can be separated from the original tissue and transferred to a separate culture, where it is able to grow independently and behaves ex-

actly as the original fragment of sarcomatous tissue, i. e. it liquefies the culture medium in the same peculiar way as did the original explanted tumor tissue by producing a very mucous secretion, and a corona of ameboid cells can be seen migrating out from the muscle, representing the same types of cells as found in the original piece.



Fig. 57.

Photo of a living culture of the Rous's sarcoma. Debris and fragments of muscle's can be seen to the left, floating in the liquefied plasma.

By and by the muscle gets thick and intransparent and debris of decayed bundles can be seen floating around together with the spherical cells in the liquefied plasma. Fig. 57. Before the muscle fragment is entirely destroyed, a new piece may be added or the old fragment divided up and subcultured with the addition of fresh muscle.

This method of cultivation of malignant cells is very simple and easy to perform. Not much training in culture technique is necessary to carry on these cells indefinitely. It is rather astonishing to see how easy these cultures are multiplied to a great extent. In a week or so, it is possible to make so many cultures, that one person would not have



Fig. 58.

Represents a section through a culture of the Rous sarcome after a couple of month's cultivation. An infiltration of the muscle fragment can be seen.

time to transfer the cultures or keep them going. It is scarcely possible to loose the strain, if nothing extraordinary happens, such as infection.

I have observed, that one single sarcoma cell is able to contaminate a piece of muscle and in as snort a

time as two days develop a culture which can be subcultured. This I have done in the following way. It is often observed, that single sarcoma cells migrate relatively long distances from the mother fragment. Under the microscope it can be seen, how such cells have migrated far out in the plasma and it is very easy to cut out that part of the plasma, containing that single cell. The plasma is then brought in contact with a fresh piece of muscle in another



Fig. 59.

Represents a section through a culture of the Rous sarcome after a couple of month's cultivation. An infiltration of the muscle fragment can be seen.

culture and it will be seen that the cells multiply very rapidly and grow into the muscle. This phenomenon will be discussed in the next chapter. It can be seen here, that it is rather simple to get pure cultures of the sarcoma cells from one single individual.

The histological examination of the cultures shows, that the muscular tissue added to the sarcomatous tissue is completely invaded by the sarcoma cells in the way typical for this tumor in vivo.

The figs 58, 59, 60 and 61 represent sections through such cultures of the Peyton Rous's sarcoma after a couple of month's cultivation. Shortly before the fixation and sectioning, a new piece of muscular tissue was added, and now it can be seen to be already invaded by sarcoma cells. In fig 58 a part of the muscle formerly added, can be seen

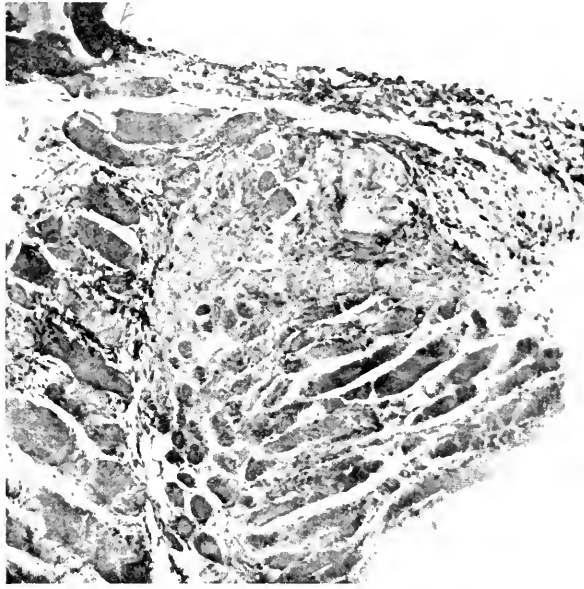


Fig. 60.

Represents a section through a culture of the Rous sarcome after a couple of month's cultivation. An infiltration of the muscle fragment can be seen.

infiltrated and overfilled with the typical polymorphous cells, characteristic for this tumor. The arrangement of the cells, bundles of fibroblast cells, crossing the field in several directions gives a picture which can be diagnosed as sarcoma

The cells seem to grow abundantly and multiply rapidly in the muscular tissue and finally substitute the muscle bundles.

The interesting fact was learned, that the sarcoma cells seem to be able to transform the protoplasm of the muscular tissue into protoplasm of their own. Only a trace of embryonic tissue juice was added to the culture medium, in order to obtain a quick coagulation and a firm clot. A whole series of cultures of sarcoma cells have been cultivated now for more than two months without the slightest embryonic tissue juice. We are now able better to understand the



Fig. 61.

Represents a section through a culture of the Rous sarcome after a couple of month's cultivation. An infiltration of the muscle fragment can be seen.

malignant, parasitic character of the sarcoma cells, when they in vivo are able to attack the normal healthy cell and destroy it.

It can now be concluded, that the muscular tissue, stored in the ice-box for a long period of time is just as good a food for the sarcoma cells as the freshly extirpated. So far it has been possible to cultivate the Rous chicken sarcoma in vitro for about 2 years with the addition of

muscular tissue, stored 2-3 weeks in the refrigerator, the mass of the tissue being increased very much during that time. It can therefore be concluded, that the sarcoma, can be cultivated indefinitely this way. The strain now under cultivation is about 2 years old.

There is every reason to believe, that other malignant tumor cells can be cultivated the same way. The human sarcomata will be tried as soon as material can be obtained.

Experiments of reinoculation of the Rous's chicken sarcoma from cultures have been undertaken all the time during the cultivation. It was observed that one single culture inoculated subcutaneously on the breast of big chickens showed to be sufficient to develop a tumor which 4-6 weeks after causes the death of the animals. Fig. 62 represents a section of a tumor from a big chicken which has been inoculated with only one culture of sarcoma cells which had undergone about 40 passages in vitro. Reinoculation of tumor tissue from cultures have been performed by Lambert and Hanes, R. H. Erdmann, but the malignant tissue had been cultivated only for a short time in vitro.

We have learned from these experiments, that it is possible to cultivate a strain of malignant tumor cells, the Rous sarcoma, indefinitely outside the body the cells still maintaining their malignant character. We are dealing with cells, which have a decidedly different physiological character than the normal tissue cells.

Numerous problems for investigation are opened up by this method. The interesting question of the relation of the stroma cells to the malignant cells, which R. H. Erdmann¹⁸³ has pointed out, will possibly be cleared up by this method, by which it is possible to obtain strains of the malignant cells from only one cell.

A method is developed, by which it is possible to cultivate sarcoma cells of the type of Rous's chicken sarcoma, indefinitely outside the body, which hitherto has been unsuccessful.

The technique is more simple than any of the others for keeping strains of tissue cells in vitro, and practically no training is necessary.

The investigation of numerous problems of importance is rendered possible by means of this method. We have here an inexpensive and simple way of carrying on the virulent sarcomatous tissue cells from piece to piece of fresh muscular tissue.



Fig. 62.

Represents a section of a tumor from a chicken which has been inoculated with a culture of sarcoma cells, that has undergone 40 passages in vitro.

By studying the Rous's chicken sarcoma in vitro according to the method already described, several new facts have been discovered already, as to the biological character of these cells. From the cultivation of the malignant sarcoma cells it is learned that these cells are able to synthesise their own living protoplasm from the serum constituents

and the dead muscular protoplasm. Only leucocytes were found to be able to build up their own protoplasm and transform the serum constituents to material which can serve as a food for fibroblasts. Fibroblasts, epithelial cells and cartilage are not able to live and multiply in media which do not contain embryonic tissue juice. In other words this means that there is a capital difference between the character of normal tissue cells and the Rous chicken sarcoma cells as types of the malignant cells. This fact helps us to understand a good deal more of the nature of the malignancy of the sarcoma cells.

Besides this character, the sarcoma cells have a remarkable ability of liquefying the plasma medium, a characteristic which has already been described long ago, and found by almost every investigator who has cultivated malignant tissue cells in vitro.

To these two biological characteristics of the malignant cells we may add a third, which, I believe, is at least equally as important as the others. By this it is possible also to explain the propagative and destructive nature of the tumor cells. The sarcoma cells of the Rous chicken sarcoma are observed to divide even when the cells are scattered, isolated and in no protoplasmic contact with other cells. In a former chapter: "The integrity of tissue cells", it was described that normal fibroblasts were not able to divide and multiply from one single cell. Only when several individual tissue cells were in close contact with one another, an outgrowth of new cells would take place. It was also mentioned that the colonising ability of normal tissue cells could not only be observed physiologically, but also histologically. Cultures of normal fibroblasts, especially after they have been stained and prepared with chloride of gold, show that all cell individuals are in anastomotic connection with one another. The tissue colony, taken as a whole resembles that of a syncytium, more than anything else.

Cultures of sarcoma cells show often a different picture. After 24--48 hours incubation, one may find sarcoma cells scattered almost all over the culture medium. This and the fact, that one single sarcoma cell is sufficient to contaminate a piece of dead muscular tissue and give rise to unlimited quantities of sarcomatous tissue, explains the relative independency of the sarcoma cells.

For the study of the cell division of the sarcoma cells, cultures were prepared of Rous chicken sarcoma and a few human sarcomata, an osteosarcoma and glio-sarcoma. The human tissues were prepared for cultures shortly after removal from the patients by operation. The culture medium for the Rous sarcoma which was found most suitable, was composed of two volumes chicken plasma and a trace of embryonic tissue juice. If too much embryonic tissue juice was added, an extensive liquefaction occurred in the plasma clot. The culture medium used for the human tissues, was composed of varying amounts of human plasma and chicken plasma and an addition of extract from the respective sarcomata or human muscular tissue.

It proved to be less difficult to obtain isolated individuals of sarcoma cells, than was the case with the fibroblasts. The observations were made during the first passages or after the sarcoma cells had been cultivated for about 14 days to 8 weeks. Usually it was not necessary to make special arrangements for getting isolated cells for the observations. The cells behaved in that respect differently from the normal fibroblasts; the tendency to form tissues is not excessive. In all the cultures it was easy to find perfectly isolated cells in the periphery of the cultures. The cells are usually very active amoeboid and migrated far out into the culture medium.

The sarcoma cells form very loose connections with other cells, compared with those of normal tissue cells and besides that, they liquefy the plasma very intensively. This was made use of in aspirating the liquefied plasma, includ-

ing the cells and expelling them on a cover-slip, on which there is a drop of fresh plasma and embryonic tissue juice. By this method, it was possible to obtain hundreds of isolated cells on one slide.

The observations were made under the high power microscope, which was placed in a special heating box, in order to keep the cultures at body-temperature during observation.

It required much patience to keep on observing these cells for hours under the high power lenses. It was very tiresome and often rather disappointing, because of the many cells and cell-groups, which were found unsuitable for these observations.

The various typical changes of the cell-shape were immediately drawn on paper. Sometimes, however, the movements were so lively, that it was almost impossible to make the drawings fast enough during the observation. In these cases, micro-cinematographic recording would have been of excellent use and would have given wonderful information.

It can be generally stated that almost in all cultures that could be observed, the sarcoma cells were able to divide independently, when they were isolated and scattered. The mechanism of the cell division of the sarcoma cells was so entirely different from that of the normal tissue cells *in vitro*, that mitotic cell division was very seldom observed in the cultures of sarcoma cells.

The cells which were seen growing out from the explanted piece of the Rous chicken sarcoma, were extremely polymorphous. The sizes of the various types of cells were innumerable. The smallest of the cells were about the size of big bacteria or blood-platelets and the largest almost 50—150 times larger than the smallest. The type of small ameboid cells and the type of the medium-sized were far the most active of all the cells. The big cells, almost giant cells, were usually very slow in movement

and preserved their shape of outline for hours. These cells looked more like big fibroblasts in respect to their spread of cytoplasm and their more or less spindle shape.

1. The type of medium and small cells.

Fig. 63 represents the type of the smallest observed cells found in the Rous chicken sarcoma in vitro. Their cytoplasm were very granulated and the nucleus could not be distinguished in the living cells. These cells were ex-

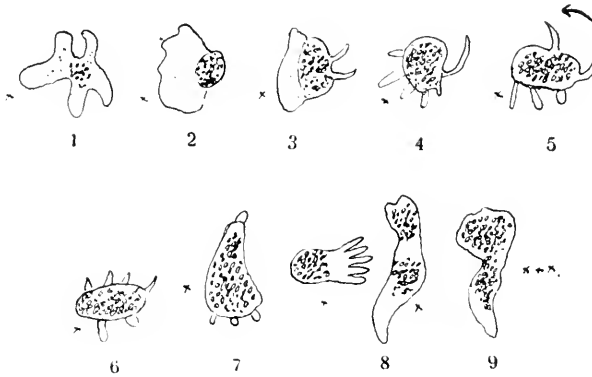


Fig. 63.

The type of the small cells in the Rous's chicken sarcoma.

tremely lively in movement. Broad tongue-like and fine filiform pseudopods were pushed out and retracted. Several filiform pseudopods could be seen put out at the same time and were observed to move like cilia, i. e., the movement, which the extreme point of the pseudopods described, was circular, concentric to the axis of the cell, figs. 63₄, 63₅, 63₆.

In fixed and stained preparations, these cells looked as shown semi-diagrammatically in fig. 64. The cytoplasm took up the stain, Azur II and methylene-blue, very intensively. The nucleus or nuclei, sometimes there were several.

were either rather distinct, fig. 64₂, or were only represented as several pieces of chromatic fragments, scattered in the protoplasm.

These kinds of cells were rather numerous represented in all the cultures of the chicken sarcoma. Besides this type, another cell type was represented, namely, a medium sized with a rather clear cytoplasm, very finely granulated and containing a few vacuoles only. Fig. 65 * represents this type. The movement of the cell was also very lively. The changes in the outline of the cell can be followed in fig. 65; the time it took for the cell to adapt the various

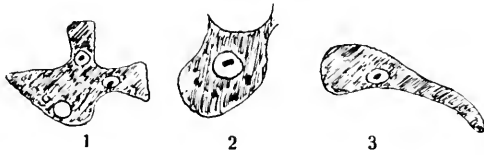


Fig. 64.

The type of the small cells in the Rous's sarcoma, stained with Azur II.

shapes, illustrated in fig. 65, was about 10 minutes. The cell, representing the medium sized type of cell, could be seen wandering in and among some other cells in the same microscopic field.

2. *The type of big ameboid cells.*

Characteristic for these cells is the slow movement of their cytoplasm. The shape is rather spherical and the cytoplasm highly granulated and the nucleus can very seldom be seen in the living cells, fig 66. When stained, the nucleus is rather distinct and usually contains one big nucleolus and a couple of smaller ones, fig. 67. Very characteristic is the peculiar way the granula and vacuoles are arranged. Often big vacuoles are arranged as a single layer in the periphery of the cell, or they are arranged as a circu-

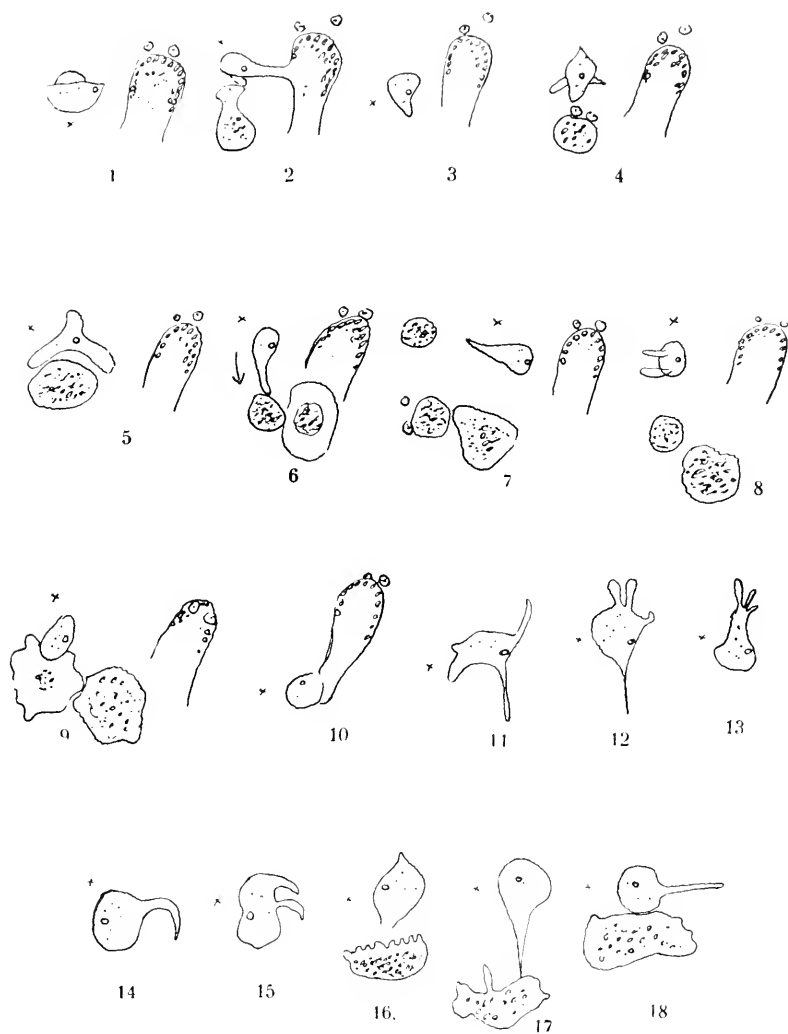


Fig. 65.

The cell marked x represent the type of the medium sized cell of the Rous's sarcome.

lar layer around the very finely granulated mass of the central part of the cell, fig. 67₂. At other times one will see colonies of even-sized vacuoles, surrounded by a thin membrane somewhere in the cytoplasm, fig. 67₁. Sometimes structures can be seen in the cytoplasm, which look almost like crystals of fatty acids.

3. *The type of spindle-shaped cells.*

These cells look almost like typical fibroblasts, but rather big. Sometimes they resemble most of all the transition forms between the big mononuclear lymphocytes and fibroblasts.

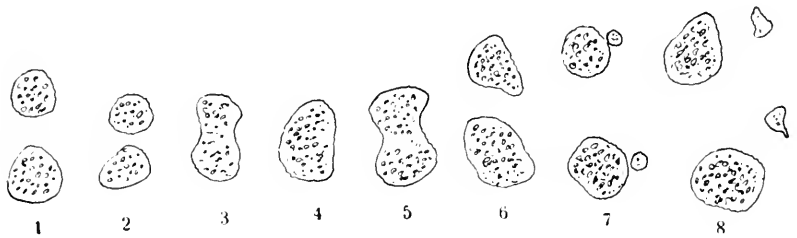


Fig. 66.

The type of big ameboid cells of the Rous's sarcome.

which are described by Carrel³⁶⁾ i. e., the one end of the cell is thin and filiform as in fibroblasts, and the other end may show filiform or tongue-like pseudopods. The cytoplasm is usually filled with granules. The nucleus may be found with a distinct membrane, containing one to four nucleoli, fig. 68. Other cells of this type may show peculiar chromatic fragments distributed in the cytoplasm, fig. 69.

Characteristic for all the cell types from the sarcomatous tissue is their great activity. Even the slow moving cells exhibit a greater motile activity than do the normal fibroblasts. Characteristic for the sarcoma cells is their ability to unite and amalgamate with other similar cells. It seems here to be a real union which takes place. It is not

a mere contact or cohesion between the cells, which so often can be observed to take place between normal fibroblasts, for instance, but a veritable amalgamation into one cell. It has so far been impossible to observe an uneven distribution of the granules of two united cells, which, on the contrary, would indicate a pseudo-amalgamation. After such an amalgamation of two or several cells has taken place, they generally separate shortly after into just as many individuals as they were before united.

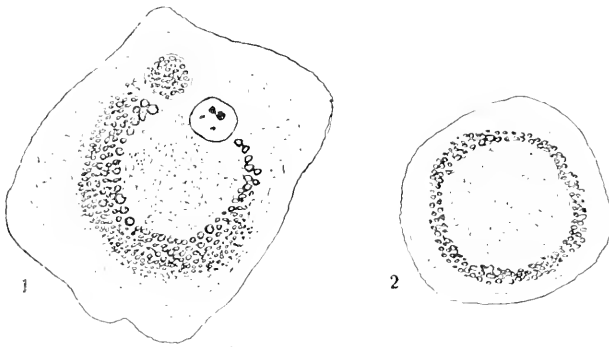


Fig. 67.

Type of the big amoeboid cells of the Rous's sarcoma stained with methylene blue.

This phenomenon of perfect amalgamation was observed in all the cultures of malignant cells, including the various sarcomata and the Flexner-Jobling rat carcinoma. It has never been observed to occur in normal fibroblasts.

Some few times, a very interesting phenomenon was observed namely, that two cells, belonging to the type of the big amoeboid cells, amalgamated perfectly, and shortly after their separation, about one to two minutes, each cell divided into two at the very same moment. The new cells were much smaller than the mother cells. This was rather a surprising fact, as it has never been observed in normal tissue cells. The observations were always made with extreme

care, in order to observe in detail what happened; and it was very easy indeed to see, that the two cells divided and that all the 4 new cells moved away in different directions with a lively protoplasmic movement. In one of the observations, it was possible to fix and stain the culture immediately after the interesting cell division and later find the cells again, thereby controlling the observation of the living cells with the same cells in fixed and stained condition. This conjugation, if we may so term it, followed by the cell division, can be seen in fig. 66.

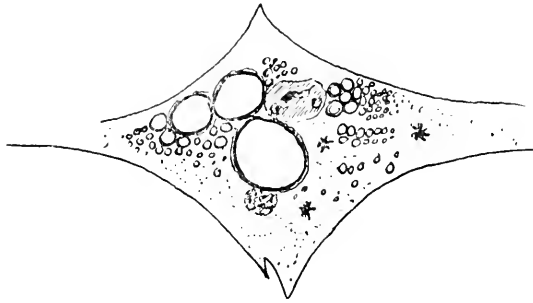


Fig. 68.

The type of spindle-shaped cells of the Rous's sarcome, stained with methylene blue.

It was frequently observed, that the isolated sarcoma cells divided independently. The new cells were very often much smaller than the mother cells. Any mitosis has not been observed. In an extreme case, one single cell separated cells which were many times smaller than the mother cell, and it was observed how one cell finally was split up into seven small cells. It is noteworthy that all the small were moving about. No wonder that the picture of the sarcomatous tissue is very polymorphous, when the cells are able to divide, separating cells of different sizes.

In some preparations a kind of struggle between two cells, not belonging to the same type, was observed. Fig. 70

illustrates how a cell, belonging to the medium-sized cell type, tried to penetrate into a much larger cell, belonging to the fibroblastic type of cell. In the fig. 70₂, 70₃, it can be seen how the small active cell is pushing against the protoplasm of the big cell. After this visit, it was interesting to discover if any changes were going to occur in the fibroblastic cell type. The only change, which could be observed, was that two vacuoles floated up to the place attacked by the smaller cell. (Fig. 70₅).

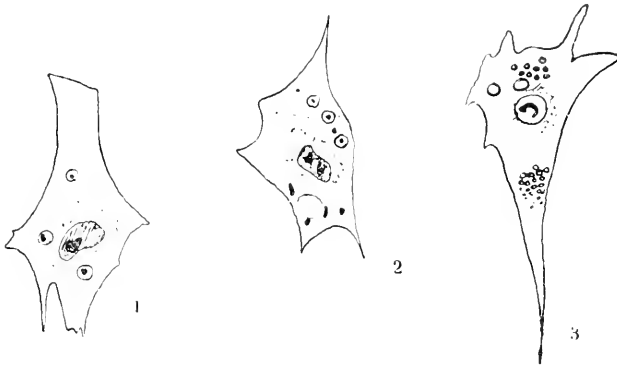


Fig. 69.

The type of spindle-shaped cells of the Rous's sarcoma stained with methylene blue.

The main conclusion of these observations was, that the cells, which are assumed to be the bearers of the malignancy, are able to divide when isolated and scattered. This is not found to be the case with isolated and scattered normal fibroblasts. The isolation and the distribution of the sarcoma cells were very easy to perform, because they form loose connections with each other. Also this indicates the independency of the sarcoma cells, compared with that of the normal fibroblasts.

Still more interesting is the fact, that the new cells, separated from the mother cells, were generally of a much smaller size than the mother cells.

In all the cultures examined, of human sarcomata, chicken sarcoma as well as of Flexner-Jobling rat carcinoma, it was observed that the cells were wandering in and among other cells, establishing perfect union with them, whereupon they separated again, wandering to other cells, uniting and separating and so on. In a few cases it was actually observed, how a perfect amalgamation of two cells took place, whereupon they separated and each cell divided and the 4 new individuals were moving around under lively ameboid movement.

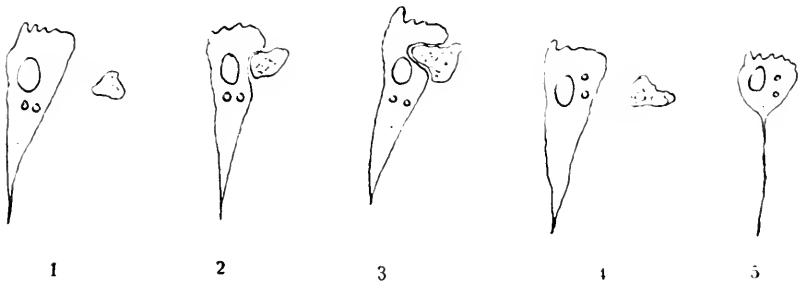


Fig. 70.

Represent a cell belonging to the medium sized cell type, trying to penetrate into a much larger cell belonging to the fibroblastic type of cell.

It is too early now to say anything about the significance of the process of amalgamation of malignant cells with regard to cell division. Compared with the observations made on normal fibroblasts, where no cell division of scattered and isolated individuals was seen, it must be said that what is described here is a general occurrence for the sarcoma cells. In almost all the examined cultures of sarcoma cells, which were isolated, cell divisions could be observed. That this is true, I found by trying to contaminate a piece of normal muscular tissue with only one sarcoma cell and I was within 48 hours able to obtain a big culture of sarcoma cells.

I may summarize the results of the experiments on the cultivation of the chicken sarcoma cells by saying:

- 1) Sarcoma cells behave as independent cell individuals, i. e. they are able to multiply even when they are scattered and isolated in the culture medium.
- 2) Sarcoma cells are able to synthesise protoplasm of their own from the serum constituents and dead muscular tissue.
- 3) Sarcoma cells liquefy the fibrin in the plasma medium more extensively than do any other normal tissue cells.

These three biological characteristics of the sarcoma cells sufficiently explain the aggressive, invasive and destructive ability of these cells *in vivo*.

In order to learn about the destructive mechanism of the sarcoma cells *in vivo*, experiments were undertaken to study the manner in which the sarcoma cells are able to attack normal tissue cells and suppress their action. We know of course that the malignant cells are able to suppress the growth of the normal tissues, but the way they do so we know absolutely nothing about.

The interrelation of normal fibroblasts and sarcoma cells was studied directly in the tissue cultures. To distinguish between the normal fibroblasts and the sarcoma cells, the latter were vitally stained.

The experiments were made in the following way: To cultures of sarcoma cells was added neutral red, trypan red or Janus green in solutions 1:20,000. At the end of 24 hours incubation, the migrated sarcoma cells had readily absorbed the stain; the cultures were then opened and by means of a fine pipette, a little of the liquefied plasma medium containing numerous vitally stained sarcoma cells, was aspirated. The contents of the pipette were introduced into a 24 hours old culture of fibroblasts (belonging to a strain 1½ year old, cultivated in the *Institute of General Pathology of Copenhagen*).

By this method I sometimes succeeded in getting a microscopical field with a vitally stained sarcoma cell in close proximity to an unstained normal fibroblast.

In the meantime the results of these experiments were rather negative. It was never learned by the method whether a sarcoma cell was able to harm a normal fibroblast in a way that could be observed directly. Experiments of this kind naturally demand a patient observation over a long period of time. If nothing happens within a relative short time, the vitally stained sarcoma cells become decolorized and no longer recognisable as sarcoma cells.

An other experimental device gave much better information.

A few carcinoma cells were added, by means of a pipette, to a big culture of fibroblasts grown in one of the culture flasks (type D) *ad modum* Carrel ⁶⁶). A few days later it was observed that the plasma in the periphery of the tissue began to liquefy, (Fig. 71). The zone of liquefaction increased in size and the colony of fibroblasts was found as a small retracted piece of tissue on the shore of the lake of liquefied plasma. At the end of three weeks or so, the entire plasma in the culture flask had liquefied.

It has been a generally opinion though not supported by experimental facts, that connective tissue cells are able to over-

grow the malignant cells in cultures. We know, furthermore, that fibroblasts can hardly be avoided when other kinds of tissue cells have to be obtained in pure culture. From experiments undertaken here, it seems as if the malignant cells are able to suppress the growth of fibro-

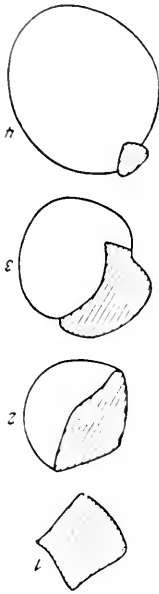


Fig. 71.
A culture of fibroblasts contaminated with a few sarcoma cells. The figures represent the various degree of liquefaction.

blasts. This is probably not due to the luxuriant growth of the malignant cells in the cultures, but because of some of the peculiarities of the malignant cells. It is without doubt the liquefying power of the sarcoma cells which has deprived the fibroblasts of their stroma, and scattered these cells. The proteolytic power of the sarcoma cells may therefore also play an important rôle in the mechanism of the malignity. That the sarcoma cells actually are able to disintegrate entirely a normal complex tissue, was demonstrated by other experiments.

To a culture of a fragment of intestine of a chicken embryo (20 days old), was added a small piece of sarcomatous tissue of the Rous sarcoma; the intestinal fragment had been cultivated *in vitro* for about a month, and intestinal epithelium had grown all around the fragment, so that it looked like an epithelial cyst. The cyst or "intestinal organism", and a piece of sarcomatous tissue was placed side by side in one culture and allowed to grow in that way. A few days later, the intestinal cyst had perfectly disappeared and the sarcomatous tissue was left. The effect of the sarcoma cells on the "intestinal organism" was a perfect disintegration of its cellular elements. The stroma of the tissue has been digested by the sarcoma cells, and the cells falling apart, could now be observed floating around in the liquefied medium. These scattered cells are no longer able to reunite or proliferate.

These biological characters observed for the malignant sarcoma cells *in vitro*, are undoubtedly responsible for their destructive, parasitic character *in vivo*.

We could imagine that the destructive process of the sarcoma cells *in vivo* would be as follows. First the stroma binding the cells together is liquefied. Consequently reorganisation cannot take place as long as the lytic agent, the sarcoma cells, is present and because the fixed tissue cells are deprived their fibrin stroma. Besides we have learned that the scattered and isolated tissue cells are not able to

grow and multiply. Furthermore normal tissue cells are not able to build up their protoplasm from the serum constituents or dead tissue cells. The sarcoma cells are, however, on the contrary able to multiply when the cells are scattered and isolated metastasis: they have the power to synthesise their own protoplasm from the serum substances or from dead tissue. On account of this it is obvious that the regeneration of normal tissue cells is handicapped.

The three factors mentioned here as the explanation of the mechanism of the malignity of the sarcoma cells, are probably not the only factors playing a rôle, but in any case they are sufficient to explain the malignity.

Another problem it would be very interesting to investigate is why the leucocytes in a case of malignant tumor, are not attracted in large quantities, secreting trephones necessary for the regeneration of the normal tissues. Probably the trephones would not have any influence on the regeneration of the normal tissues, even if there were plenty of leucocytes, because of the liquefaction of the fibrin stroma which is caused by the malignant cells. It should be indicated here, that the proteolysis caused by the malignant cells are probably also responsible to some extent for the metaplasia of these cells. According to the experiments of Uhlénhuth¹³⁹ and others, we know how the morphology of the tissue cells depends upon the physical conditions of the milieu extérieure.

It has been stated by several investigators that certain tissues or organs are spontaneous or primary resistant to malignant tissue growth. It has been stated by Murphy¹⁴⁷ that spleen tissue, for instance, has an inhibiting effect on the growth of malignant tumors.

I have therefore tried to add various organs to the cultures of Rous sarcoma instead of the muscular tissue. So far the sarcoma cells have been able to invade, during the process of destruction and disintegration, the various tissues. It was found that spleen had a marked accelerating in-

fluence on the rate of growth and on the malignity of the sarcoma cells.

Numerous problems are being worked out at the present time to investigate the behaviour of sarcoma cells towards normal tissue cells: and as it has often been observed that some chickens are primarily immune to inoculation of the Rous sarcoma, it is my hope by means of the tissue cultivation method to discover whether this immunity is humeral or cellular.



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